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UNIVERSITY OF ALBERTA

GENERATION AND CHARACTERIZATION OF SIDEROPHORE-DEFECTIVE MUTANTS OF *AZOTOBACTER VINELANDII*

BY

Mehmet Serdal Sevinç

A thesis submitted to the Faculty and Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

Edmonton, Alberta
SPRING, 1992

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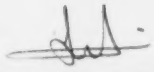

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled GENERATION AND CHARACTERIZATION OF SIDEROPHORE-DEFECTIVE MUTANTS OF *AZOTOBACTER VINELANDII* submitted by MEHMET SERDAL SEVINÇ in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

Supervisor, Dr. W.J. Page

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Date: December 2, 1991

ABSTRACT

Mutagenesis of the genome of *Azotobacter vinelandii* with a Tn*5luxAB* construct resulted in the generation of a number of siderophore-defective mutants. The iron-regulated bioluminescence encoded by the promoterless *luxAB* genes on the construct indicated that the mutations were in iron-regulated genes. Among a number of iron-regulated mutants only a few were identified as being defective in siderophore production. Characterization of these mutants showed that strain D27 was azotobactin siderophore-minus, did not produce bioluminescence, but its catechol siderophore production was not affected. Strain F196 was defective in the production of 2,3-dihydroxybenzoic acid (2,3-DHBA) and the siderophores derived from 2,3-DHBA, namely azotochelin and aminochelin, but produced iron-regulated bioluminescence, and 2.5 times as much azotobactin as the parent strain. A mutant strain P100 that is defective in known siderophores was generated by transforming the DNA from strain F196 into another spontaneously obtained azotobactin-minus strain (UA1). In contrast to other siderophore-minus strains, strain P100 was unable to grow when the synthetic iron-chelator ethylenediamine-*N,N'*-bis 2-hydroxyphenyl acetic acid (EDDHA; 50 μg/ml) was included in the iron-deficient plate medium unless the organism had an adequate supply of stored iron. This inhibitory effect of EDDHA was also seen in iron-sufficient liquid medium. In both cases this inhibition was relieved by exogenously supplied azotobactin and 2,3-DHBA-derived siderophores, but not by 2,3-DHBA itself.

However, strain P100 also gave an iron-repressible positive reaction in the chrome azurol-S assay (CAS), a non-specific test for siderophores. Strain P100 was also able to grow and accumulate more iron from the insoluble iron-containing minerals FeS, vivianite and Fe_3O_4 than was obtained by simple diffusion. Furthermore, strain P100 was able to accumulate much larger amounts of internal iron than the parental strain when grown with ≥ 200 μM iron. Analysis of the highly concentrated culture supernatants of strain P100 with a combination of ethyl acetate extraction, gel exclusion chromatography and the CAS assay showed the presence of three new iron-chelators. One compound in the aqueous phase had a non-conventional structure and the largest molecular size among the three. The

other two had catechol structures, but were not derived from 2,3-DHBA, were found in the ethyl acetate phase. Furthermore, a reducing activity appeared to associate with these three compounds.

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ABBREVIATIONS

2,3-DHBA	2,3-dihydroxybenzoic acid
DBS	2,3-dihydroxy-*N* -benzoyl-L-serine
EDDHA	ethylenediamine-*N,N'* -bis 2-hydroxyphenylacetic acid
CAS	chrome azurol S
IROMPs	iron-regulated outer membrane proteins
TLC	thin layer chromatography
TSA	total siderophore activity
MW	molecular weight

1. INTRODUCTION

1.1. Biological importance of iron

Iron is the fourth most abundant element in the biosphere, following O > Si > Al > Fe (Neilands *et al.*, 1987). Being abundant as well as its involvement in many biological processes makes it a very important part of many biological systems. During the course of evolution, almost all living forms of life have evolved to accommodate this element in their structure. Subsequently iron found itself in the compartments of many subcellular units of the cell. These included many biologically vital proteins, generally enzymes of electron transport system, such as cytochromes (Slater, 1987), ferrodoxins (Matsubara *et al.*, 1987), flavoproteins (Neilands, 1974), hydrogenases (Stam *et al.*, 1987), nitrogenases for nitrogen-fixing organisms (Cammack, 1988; Haaker & Klugkist, 1987); oxygenases (Nozaki & Ishimura, 1974); enzymes of cell defence system against oxygen toxicity, such as catalase (Yonetani, 1974), and superoxidase dismutase (Steinman, 1982); oxygen transport proteins, such as leghaemoglobin of *Rhizobium* spp. (Bisseling *et al.*, 1986; Godfrey *et al.*, 1975), and storage proteins, such as bacterioferritin (Harrison *et al.*, 1987).

1.2. Iron in ecological and cellular environments

Despite the demand of many organisms for iron, its availability is restricted for biological absorption in the aerobic environment and at neutral pH because of its oxidation resulting in its low solubility (10^{-18} M) and tendency to form high molecular mass colloids (Spiro & Saltman, 1969, 1974). However, in anaerobic environments and at acidic pH, its solubility greatly increases (10^{-1} M). Furthermore, in the latter case, iron is found in the reduced, ferrous state, but with oxidation and increase at pH to 7.0 the oxidized state of the ion is favoured (Reed, 1982; Spiro & Saltman, 1974; Subcommittee on Iron, 1979). These two stable biological redox states of iron have played an important role in the evolution of iron-uptake systems of micro-organisms.

Intracellular environments also restrict the availability of iron by linking it to proteins (Critchton & Charloteaux-Wauters, 1987; Neilands, 1974, 1980), and thus

minimizing its free availability (Williams, 1982; Harrison *et al.*, 1987). This process also prevents its undesirable interactions with oxygen that generates highly toxic free radicals from superoxide and hydrogen peroxide (Wrigglesworth & Baum, 1980; Dunford, 1987) which react with proteins, DNA and other vital parts of the cell (Halliwell & Gutteridge, 1986; Wolff *et al.*, 1986; Neilands, 1989)

1.3. Evolution of iron uptake systems.

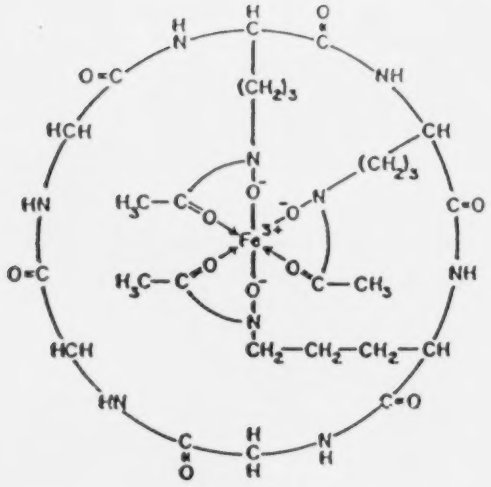
During the course of evolution, the biosphere of the Earth showed a transition from anaerobiosis to aerobiosis. With the emergence and accumulation of molecular oxygen, acquisition of this most abundant element by living things became a crucial step for survival under aerobiosis (Neilands, 1972; Byers, 1986). For this reason, only those cells that evolved the most efficient systems for iron acquisition survived best under aerobiosis. However, some species also appeared to replace iron partially or almost completely with other cofactors or ions notably Mn^{2+}. The organisms belong to the latter group mainly gather in the genus *Lactobacillus* (Archibald, 1983; Neilands, 1981a). Thus, iron demands of micro-organisms showed a great diversity from the start.

Because iron is often found in Fe^{3+} form in aerobic environments, most micro-organisms evolved to produce ligands to chelate Fe^{3+} and bring it into the cell. These chelators are called "siderophores" and appear to be the major components of high-affinity iron uptake systems. Bonding of the ligands with iron occurs often through oxygen in the structure of the ligand groups. Oxygen atom is like iron since it has six octahedrally-directed valence bondings for mutual attachment. During formation of the bonding between iron and the ligands, H_2O or OH^- ions found in the coordination sphere of iron are replaced with oxygen (Charley *et al.*, 1963; Spiro & Saltman, 1974). However, in some cases, the bonding between iron and the ligands are achieved through oxazoline nitrogen atoms instead of oxygen.

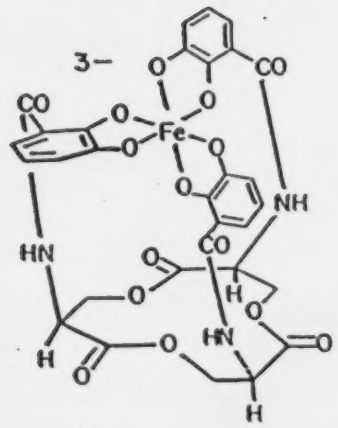
1.4. Siderophores

Siderophores are low molecular weight (MW) compounds (500-1,000 Daltons) released into the environment in order to chelate iron and bring it,

specifically Fe^{3+}, (affinity for Fe^{3+}, $K_f > 10^{30}$) into the cell. This observation was first made in *Ustilago sphaerogena* by Neilands (1952). Thus, ferrichrome was the first isolated siderophore (Fig. 1). It was later characterized as a cyclic hexapeptide ferric trihydroxamate (Garibaldi & Neilands, 1955). Later, another other type of siderophore, enterobactin (enterochelin) was isolated first from *Salmonella typhimurium* (Pollack & Neilands, 1970), and then from *Escherichia coli* (O'Brien & Gibson, 1970) and other members of the *Enterobacteriaceae* such as *Enterobacter, Shigella* and *Klebsiella*. Enterobactin is a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine (Fig. 1), a derivative of 2,3-dihydroxybenzoic acid (2,3-DHBA). In *E. coli* 2,3-DHBA is synthesized from chorismic acid in three steps (Fig. 2). Because of DHBA functional groups, this was the first example of catecholate/phenolate type siderophores while ferrichrome was the first known hydroxamate type siderophore. Thus, siderophores were first classified into two major functional groups: hydroxamate types and catecholate/phenolate types. The former group appears to be common in both bacteria and fungi while the latter appears to be unique to bacteria. Both of these groups also contain additional liganding groups, such as alpha-hydroxy acid (e.g. aerobactin by *Aerobacter aerogenes*, Gibson & Magrath, 1969), oxazoline (e.g. mycobactins by *Mycobacterium* spp., Snow, 1965a, 1965b), thiazoline (e.g. pyochelin by *Pseudomonas aeruginosa*, Cox *et al.*, 1981), fluorescent quinolinyl (e.g. pyoverdin by Pseudomonads, Meyer & Abdallah, 1978). Despite these conventional siderophore structures, there are also some siderophore structures that do not fall into either of the catecholate or hydroxamate groups. For example, citrate either by itself (Guerinot *et al.*, 1990) or as a functional group of some hydroxamate siderophores like aerobactin, arthrobactin of *Arthrobacter* spp. (Lochhead & Burton, 1953), schizokinen of *Bacillus megaterium* (Garibaldi & Neilands, 1956; Lankford *et al.*, 1966; Byers *et al,* 1967) plays a siderophore-like role in the process of iron uptake. Other non-conventional siderophores include rhizobactin from *Rhizobium meliloti* (Smith & Neilands, 1984, 1987; Smith *et al.*, 1985) and staphyloferrin by *Staphylococcus hyicus* (Meiwes *et al.*, 1990) that have carboxylic acid functional groups, and alpha-hydroxyisovaleric acid from *Proteus mirabilis* (Evanylo *et al.*, 1984).



Ferrichrome



Enterobactin

Figure-1. Examples of hydroxamate and catecholate-type siderophores in their ferrated state (ferrichrome, adapted from Lankford, 1973; enterobactin, adapted from Neilands *et al.*, 1981).

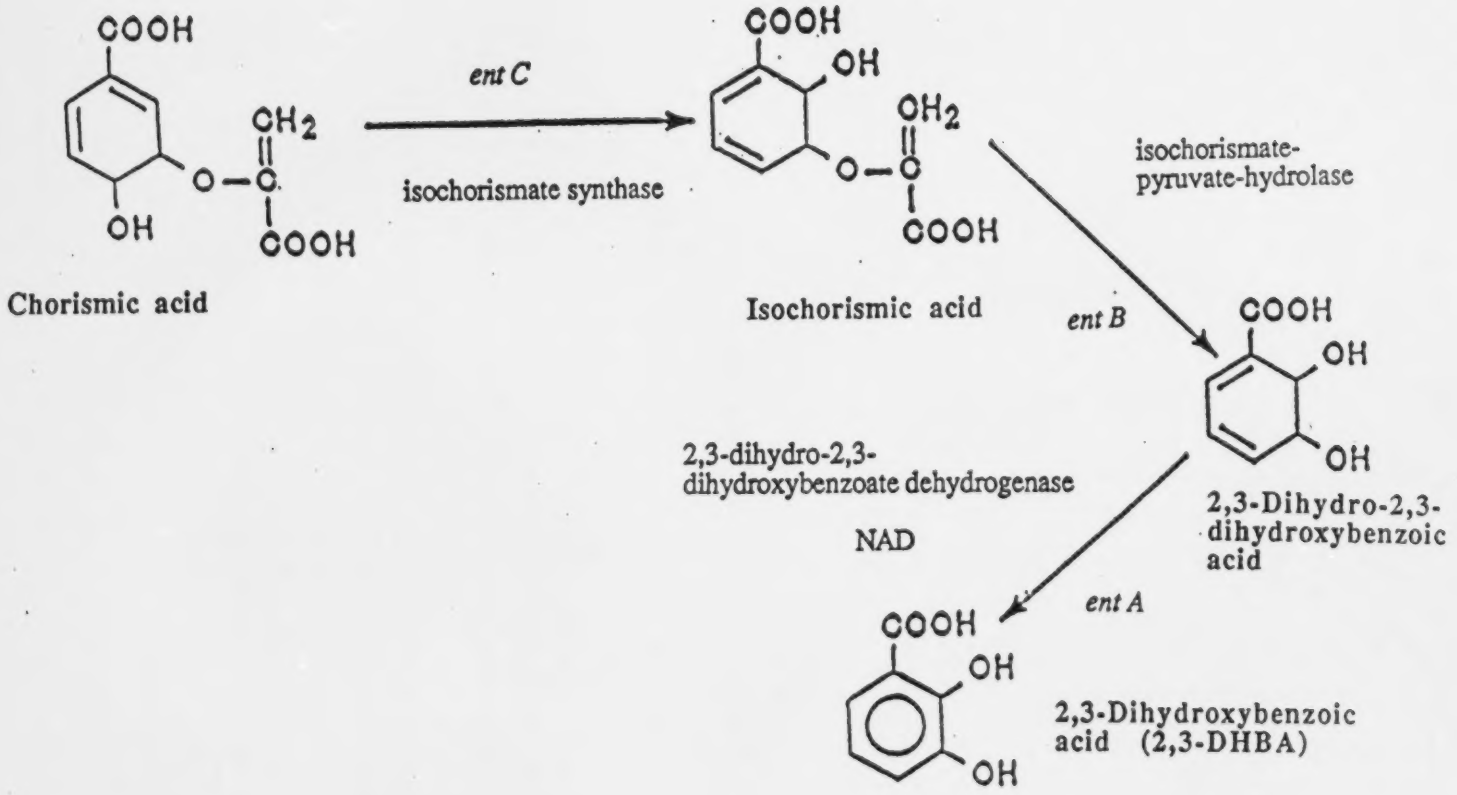


Figure-2. Biosynthetic pathway for 2,3-DHBA synthesis in *Escherichia coli*. 2,3-DHBA is synthesized from chorismic acid in three steps in the pathway of enterochelin synthesis (adapted from Earhart, 1987).

These compounds are produced by most aerobic, facultative anaerobic bacteria and fungi including phytopathogens (Leong & Neilands, 1982; Enard *et al.*, 1988), animal and human pathogens (Chart & Trust, 1983; Holzberg & Artis, 1983; Griffiths *et al.*, 1988). Nevertheless, certain *Lactobacilli* do not appear to produce these compounds. However, there are also some other micro-organisms that need iron but they do not seem to produce siderophores: these are mainly *Legionella* spp. (Reeves *et al.*, 1983) *Yersinia* spp. (Perry & Brubaker, 1979), *Neisseria* spp. (West & Sparling, 1985), *Listeria monocytogenes* (Cowart & Foster, 1985), *Streptococcus mutans* (Evans *et al.*, 1986) and *Saccharomyces cerevisiae* (Neilands *et al.*, 1987). Of which, *Niesseria* spp. are known to acquire iron by direct cell contact from transferrin (Griffiths *et al.*, 1988; Mc Kenna *et al.*, 1988), while *Y. pestis* is able to utilize the iron from myoglobin, hemoglobin and ferritin but not transferrin or lactoferrin as a result of expression of iron-regulated outer membrane proteins with the concomitant expression of iron-regulated outer membrane proteins (IROMPs) (Sikkema & Brubaker, 1989). *S. mutans* efficiently utilizes Fe^{2+} through a transmembrane electrochemical gradient (Evans *et al.*, 1986). On the other hand, *S. cerevisiae* can acquire iron by acidifying the growth medium, possibly by producing citrate (Neilands *et al.*, 1987).

Although siderophores are specific for Fe^{3+}, some other metal ions can also bind to siderophores, such as trivalent ions Al^{3+} (Hider, 1984; Neilands, 1981b, 1984a), Ga^{3+} (Emery & Hoffer, 1980; Emery, 1987), Cr^{3+} (Neilands, 1984a), divalent ion Cu^{2+} and anion MoO_4^{2-} (Hider, 1984). However, these bindings are not expected to play a significant role in nature.

Furthermore, production of siderophores may also be induced under iron-sufficient conditions by using metal ions such as Co^{2+} in *Neurospora crassa* (Padmanaban & Sarma, 1966). The presence of Co^{2+} in the environment blocks iron incorporation into heme, and subsequently causes an increase in the production of hydroxamate siderophores. In *Ustilago sphaerogena*, the induction of siderophore production by excess Co^{2+} is shown to be reversed by another metal, Zn^{2+} (Komai & Neilands, 1966). The latter metal, Zn^{2+}, also is shown to increase siderophore production in *Azotobacter vinelandii* (Huyer & Page, 1988).

Induction of siderophores in *A. vinelandii* by Zn^{2+} results in the inhibition of a ferric reductase rather than competition for siderophore binding.

1.5. Determination of siderophores

Over the years many techniques for siderophore analysis have been developed. Siderophores are now relatively easily detected, and quantification of the functional groups can be carried out as a function of their concentration (Neilands, 1984b, 1989). Other than membrane-bound mycobactins (Ratledge, 1987), most siderophores are normally found in the cell culture supernatants. Siderophores can be determined with general assays as well as specific assays for the functional groups. Generally most siderophores form coloured complexes with Fe^{3+} (Neilands, 1984b, 1989). Their fluorescent groups also can be visualized under short wave (254 nm) and long wave (366 nm) UV light. The CAS (chrome azurol S) assay developed by Schwyn & Neilands (1987) is a significant step for detection of siderophores. This test is a general, diagnostic assay for siderophore production that is not dependent on the structure of the siderophore ligand. This gives an easy detection method that can be used in the agar media as well as cell-free solutions. In the CAS assay, chrome azurol S makes a complex with Fe^{3+} and a detergent, giving a blue complex. The affinity of CAS for Fe^{3+} is less than most siderophores, so that siderophores can quantitatively capture Fe^{3+} from the CAS-Fe^{3+} complex, decolorizing the complex to an orange colour (Schwyn & Neilands, 1987; Neilands, 1989).

Specific assays include the detection and quantification of catecholate or hydroxamate groups. When scanned spectrophotometrically, most siderophores give peaks, such as the catecholates around 315-320 nm (Neilands, 1989). Over the years, although a number of assays have been developed for detection of these specific groups, only some of them are used routinely. Hydroxamates are best determined by the Csaky assay (1948). This assay is based on iodine oxidation of hydroxylamines to nitrite following acid-heat hydrolysis of the *N* '-substituted hydroxylamine group of the hydroxamate siderophores (Gillam *et al.*, 1981). Catecholates are best determined by the method of Arnow (1937) or its improved form by Barnum (1977) that determines vicinal aromatic hydroxyl groups. This is

based on nitration of the aromatic ring with sodium nitrite and following conversion of it to a molybdate complex with sodium molybdate, that is yellow when acidic but turns to red with the addition of NaOH.

Other than physical and chemical methods cited above a synthetic chelator, EDDHA (ethylenediamine-*N,N'* -bis 2-hydroxyphenylacetic acid), can be included in the medium in order to detect production of siderophores by micro-organisms. EDDHA can bind Fe^{3+} strongly, but siderophores can deferrate the EDDHA-Fe^{3+} complex because they have higher affinities for Fe^{3+} than EDDHA has. Furthermore, EDDHA is not normally utilized as a nutrient by bacteria (Neilands, 1989). Thus, the EDDHA test can be used as a bioassay to detect siderophore production. The biological assays are often useful as general assays for initial detection of siderophores prior to determination of their structure.

1.6. Iron demand of micro-organisms

While *Lactobacillus* can survive with below the current detection limit of iron, some organisms like magnetotactic bacteria demand excessive amounts of iron, up to 1.5 % of their dry weight is magnetite (Frankel *et al.*, 1979). However, in most micro-organisms iron constitutes less than 0.1 % of the dry weight (Lankford, 1973; Neilands, 1974). Iron requirements of micro-organisms in the medium generally vary as follows: the range for Gram-negative bacteria is usually 0.36 μM for enterics and 1.6 μM for aerobes (Lankford, 1973; Waring & Werkman, 1942); Gram-positive bacteria and fungi have a wider range from 0.4-4.0 μM (Weinberg, 1974). Iron requirements of micro-organisms may also be affected by external conditions such as carbon source (Meyer & Abdallah, 1978; Neilands, 1984b), nitrogen source (Carnahan & Castle, 1958; Esposito & Wilson, 1956; Subramanian *et al.*, 1968), the form of iron and the mechanism of uptake used by the organism (Pollack *et al.*, 1970). Although overall demands vary, usually a 10 μM concentration in the medium is assumed iron-sufficient for optimum growth while a 0.1 μM concentration is iron-deficient (Neilands, 1984a).

1.7. Iron-regulated outer membrane proteins (IROMPs)

The water-filled pores lined with porin proteins found in the outer membrane of Gram-negative bacteria can efficiently facilitate the passage of amino acids, sugars, and other small molecules while they fail to transport siderophores since the size exclusion limit of outer membrane porins cannot accommodate ferric-siderophore complexes (Neilands, 1982; Nikadio & Vaara, 1985). Specific receptor proteins have evolved for the transport of ferric siderophores into the cell. The receptor proteins of Gram-negative bacteria for ferric-siderophore complexes are usually found in the outer layer of cell envelope. Currently, little is known about the receptor proteins of fungi and Gram-positive bacteria. However, the presence of iron-regulated envelope proteins ranging from 25 kiloDalton (kDa) to 180 kDa was demonstrated in *Mycobacterium smegmatis* (Hall *et al.*, 1987). Unlike the Gram-negative bacteria, iron regulated receptor proteins of fungi are found in the cytoplasmic membrane (Winkelman & Huschka, 1987; Winkelman & Braun, 1981; Adjimani & Emery, 1988).

Because most of the information has been derived from Gram-negative bacteria, and these proteins are found in the outer membrane of these organisms, they will be referred as IROMPs. The synthesis of induced proteins under iron-deficiency have been well studied in *E. coli* and their presence later has also been demonstrated in a variety of organisms including the ferrichrome receptor proteins of *E. coli* (Hantke & Braun, 1975) and *S. typhimurium* (Braun *et al.*, 1977; Luckey & Neilands, 1976). Later, the receptor protein for enterobactin was identified (Hancock & Braun, 1976) and its specificity for enterobactin was studied (Ichihara & Mizushima, 1977; Hollifield & Neilands, 1978). A similar specific relationship between a ferric-siderophore and its receptor was also shown in some strains of *Pseudomonas* that produce pyoverdins (Hohnadel & Meyer, 1988; Magazin *et al.*, 1986). However, despite this specificity some organisms can either directly or indirectly utilize siderophores excreted by other organisms. Thus *E. coli* can directly utilize fungal siderophore ferrichrome because the organism has the IROMP for ferrichome; however after internalization of ferrichrome-Fe^{3+} complex, ferrichrome is released from the cell into the environment with a lowered affinity for iron (Hartmann & Braun, 1980). Indirectly, siderophores excreted by *Azotobacter*

vinelandii promote the growth of *Agrobacterium tumefaciens* (Page & Dale, 1986). The growth promotion of *Agrobacterium tumefaciens* is believed to be the result of scavenging iron from the *Azotobacter* siderophores (Page & Dale, 1986). However, the experiments demonstrating the utilization of siderophores by other organisms are technically difficult since it is easier to label the iron rather than the siderophore itself (Emery, 1987).

Most IROMPs of Gram-negative bacteria have molecular weights in the range of 60-90 kDa (Neilands, 1982). In addition to their involvement in the uptake of ferric-siderophore, they may also be the targets of certain lethal agents, like bacteriocins, bacteriophages, and antibiotics (Neilands, 1989). This is of significant interest to the process of evolution since these lethal agents can efficiently compete with the siderophores for the same receptor. Furthermore, a number of IROMPs of *E.coli*, like FhuA for ferrichrome, FepA for enterobactin, Iut for aerobactin, FhuE for rhodotorulic acid and coprogen and FecA for citrate have been analyzed. It was found that these proteins show similarity with the porin proteins in the outer membrane, including a signal sequence, similar acidic net charge, lack of extended regions of hydrophobicity and alpha-helical regions. However, they have a short sequence of their N-terminal region not shared with other outer membrane proteins (Krone *et al.*, 1985; Coulton *et al.*, 1986; Lundrigan & Kadner, 1986; Braun *et al.*, 1987; Sauer *et al.*, 1987; Pressler *et al.*, 1988; Nau & Konisky, 1989). The last variation may aid their interaction with the TonB protein (Lundrigan & Kadner, 1986), that is anchored in the cytoplasmic membrane but faces the periplasmic space and facilitates the transport of ferric-siderophore in an energy dependent manner (Braun *et al.*, 1987; Braun, 1990).

IROMPs together with siderophores form the major components of the high-affinity iron uptake system. This system also includes mechanisms for the release of iron from the ferric-siderophore complex. Release mechanisms vary from one organism to another as well as from one type of siderophore to another. However, the process generally involves various enzymes that release the iron from the complex by hydrolysis of the ligand and/or reduction (Arceneaux, 1983; Emery, 1987), so that reduced iron can be used by the cell.

Rhodotorulic acid of *Rhodotorula pilimanae* (Carrano & Raymond, 1978) and ferrichrome of *U. sphaerogena* (Crichton & Charloteaux-Wauters, 1987) release their iron on the cell surface and are recycled (taxi-cab mechanism). On the other hand, schizokinin of *B. megaterium* and enterobactin of *E. coli* release their iron inside the cell. During the dissociation process, schizokinin is modified or destroyed (Arceneaux *et al.*, 1973) and enterobactin is hydrolyzed to 2,3-dihydroxybenzoyl serine (Rosenberg & Young, 1974). Furthermore, when ferrichrome is used by *E. coli* during iron transport, it is found to be acetylated and returned to the medium with a lowered affinity for iron (Hartmann & Braun, 1980).

In addition to siderophore mediated high-affinity iron uptake systems, acquisition of iron under iron-sufficient conditions is attributed to the operation of a low-affinity iron uptake system. Currently, little is known about the operation of low-affinity iron uptake system. However, its existence is certain from the fact that mutants that are deficient in siderophore(s) production are viable when maintained on iron-rich conditions (Pollack *et al.*, 1970; Frost & Rosenberg, 1973; Neilands, 1977, 1981b, 1984a; Stuart *et al.*, 1980).

1.8. Regulation of iron uptake at the molecular level

Recently, with the advent of molecular biology, a new era has started in the study of iron metabolism. As would be expected, most of the work concerning regulation of iron uptake has been carried out in *E. coli*. The *fur* (ferric uptake regulator) gene of *E. coli*. is involved in the regulation of iron uptake (Hantke, 1981). The Fur protein is the *fur* gene product (17 kDa), and it binds to DNA at a specific consensus sequence, the so called "iron box", consisting of about 20 base pairs of sequence in the operator region of iron-regulated genes (Schaffer *et al.*, 1985; Bagg & Neilands, 1987; Calderwood &Mekalanos, 1988; De Lorenzo *et al.*, 1987). The Fur protein acts as a *trans*-acting protein, interacting with Fe^{2+} and negatively regulating iron uptake and/or iron regulated genes at the transcriptional level (Worsham & Konisky, 1981; Fleming *et al.*, 1983; Hantke, 1984; Neilands, 1989).

Studies show that the genes involved in iron uptake are clustered in a large operon (Neilands, 1984a), and the system is coordinately induced or repressed,

depending on the available iron (McIntosh & Earhart, 1977; Hantke, 1981). In a recent study, because of its relatively simple structure aerobactin consisting of citrate, N^6-hydroxylysine, and acetate was cloned. The results showed that the genes for synthesis and transport of aerobactin are organized in an operon, consisting of four genes for biosynthesis (*iucA, B* two subunits of the synthetase, *iucC*, an acetylase, *iucD*, an oxygenase) and one gene (*iutA*) for transport (De Lorenzo & Neilands, 1986; Neilands, 1989). These genes were transcribed in the order of *iucA, B, C, D,* and *iutA* and their molecular weights were 63, 33, 62, 53 and 74 kDa, respectively. The last protein was initially identified as the IROMP for aerobactin. Characterization of the aerobactin operon has been of significance since this is the first example of a well studied iron regulated operon at the molecular level (Neilands, 1989).

In addition to Fur-regulated iron uptake, increasing temperature, has also been noted to affect siderophore production by decreasing the level of the production (Garibaldi, 1971; Ismail *et al.*, 1985; Worsham & Konisky, 1984). However, it is apparent that the effect of temperature is an external factor and not related to intrinsic Fur-directed regulation.

1.9. Medical importance

Some siderophores called sideromycins, like ferrimycins and albomycins, act as chemotherapeutic agents (Winkelmann, 1986). However, their therapeutic use is restricted because of high frequency of resistance development.

Only one siderophore from *Streptomyces pilosus* is commercially available for therapeutic use, Desferrioxamine B (Desferal®-Ciba-Geigy) is used for treating iron overload cases (Subcommitee on Iron, 1979; Raymond *et al.*, 1984), although this is limited, currently, due to possible complications such as chemical lability, low solubility and low residence span in the body (Hider, 1984; Raymond *et al.*, 1984), and possible promotion of the growth of pathogens (Rogers *et al.*, 1984; West & Sparling, 1987). There are possibilities for using siderophores for treating poisoning with toxic metals like Al^{3+} and plutonium, (Hider, 1984). The fungal siderophore, hadacidin, has long been known to have an antitumor activity (Kaczka

et al., 1962). Siderophores may also be used to prevent formation of free radicals *in vivo* (Wilson, 1977; Halliwell & Gutteridge, 1986).

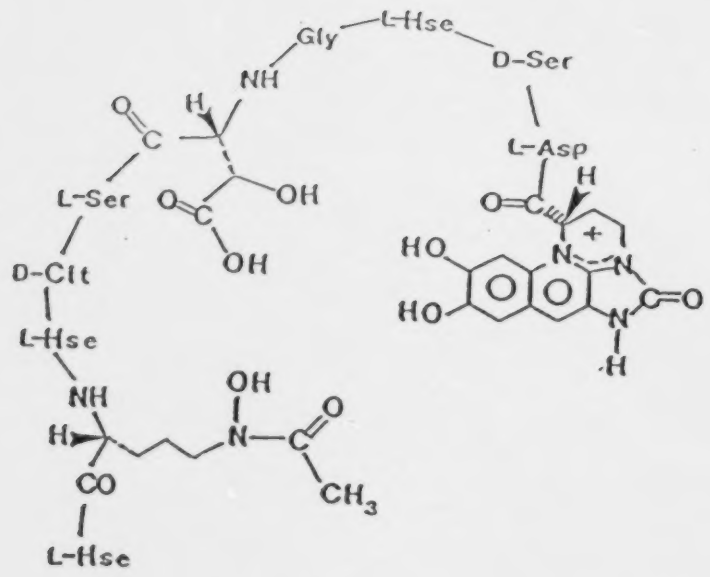
Furthermore, IROMPs are the target of vaccine production studies because they are potent antigens of most pathogens. Most pathogens in the body experience iron-deficiency due to an almost complete absence of free iron in the body and production of IROMPs always appear to be expressed during the infection. Some siderophores act as virulence factors, such as amonabactin by *Aeromonas hydrophila*, aerobactin by *E. coli*, anguibactin by *Vibrio anguillarum* (Byers, 1987; Crosa, 1987).

1.10. Agricultural importance

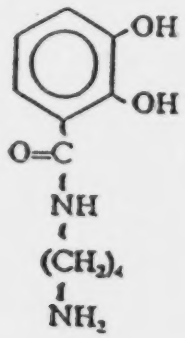
Siderophores secreted by some plant pathogens may be virulence factors as observed with pathogens infecting animal hosts (Neilands, 1989). For example, a catecholate siderophore from *Erwinia chrysanthemi*, a phytopathogen which infects a variety of monocotyledonous and dicotyledonous plants, is involved in the virulence of the organism (Expert & Toussaint, 1985). Flourescent *Pseudomonas* species that produce pyoverdin siderophores may inhibit the growth of pathogens in the rhizosphere and enhance crop production (De Weger *et al.*, 1987). Eventually these micro-organisms may be used as biopesticides (Buyer & Leong, 1986; Leong, 1986). Further research will probably increase the importance of siderophores in agriculture and related environmental studies.

1.11. Goals of the thesis

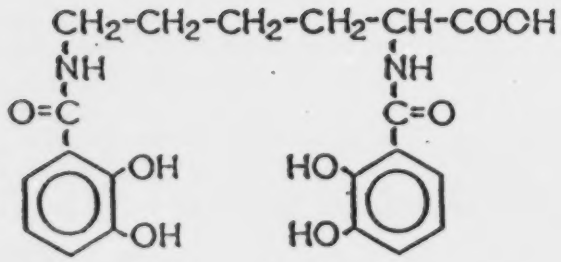
The organism of interest is *Azotobacter vinelandii* that is a Gram-negative, obligate aerobic, nitrogen fixing, soil organism (Thompson & Skerman, 1979). Upon growth in iron-deficient conditions, *Azotobacter vinelandii* produces one pyoverdin-type siderophore called azotobactin (Fig. 3) (Demange *et al.*, 1986, 1987; Page *et al.*, 1991). Azotobactin consists of a yellow-green fluorescent chromophore and a linked peptide. The composition of azotobactin is as follows: Chromophore-L-aspartate-D-serine-L-homoserine-glycine-hydroxyaspartate-L-serine-D-citrulline-L-homoserine-N^{∂}-acyl-N^{∂}-hydroxyornithine (formyl or acyl)-homoserine. Moreover, within the peptide a hydroxamate group is contributed by



Azotobactin



Aminochelin



Azotochelin

Figure-3 Siderophores produced by *Azotobacter vinelandii* : azotobactin (from Demange *et al.*, 1987), azotochelin (from Page & Huyer, 1984), and aminochelin (from Page & von Tigerstrom, 1988).

N^{∂}-acyl-N^{∂}-hydroxyornithine, thus azotobactin is a hydroxamate-type siderophore. At the same time, however, because of the containment of a chromophore part within its structure, azotobactin is also known to be a pyoverdin-type siderophore. *A. vinelandii* also produces two 2,3-dihyroxybenzoic acid (DHBA) derived catecholate-type siderophores called azotochelin: 2-*N*, 6-*N* -di (2,3-dihyroxybenzoyl)-L-lysine (Corbin & Bulen, 1969) and aminochelin: 2,3-dihyroxybenzoylputrescine (Page & von Tigerstrom, 1988) (Fig. 3). In addition, 2,3-DHBA (Fig. 2) itself is found in the iron-deficient and iron-sufficient culture fluids (Corbin & Bulen, 1969), and is assumed to be involved in low-affinity iron uptake (Page & Huyer, 1984). When the supernatant of *A. vinelandii* is extracted with ethyl acetate, azotochelin and 2,3-DHBA pass into the ethyl acetate phase while aminochelin and azotobactin are found in the aqueous phase. The pyoverdin and catecholate siderophores of *A. vinelandii* are produced in a sequential manner: when the iron concentration of the growth medium drops to about 7 μM Fe^{3+}, production of catecholate-type siderophores starts; and with a further decrease, to about 3 μM Fe^{3+}, production of azotobactin starts (Page & von Tigerstrom, 1988). Siderophore production is accompanied with the production of four IROMPs, 93 kDa, 85 kDa, 81 kDa, and 77 kDa (Page & von Tigerstrom, 1982; Page & Huyer, 1984).

The aim of this work was to study iron regulation and related siderophore functions in an attempt to bring new insights into the regulation of iron metabolism. In order to study iron uptake and regulation in *Azotobacter vinelandii*, mutants defective in iron-regulated phenotypes have been developed by transposon mutagenesis. The genome of the organism was mutagenised with a Tn*5*-derived transposon, Tn*5luxAB* (Berg *et al.*, 1989). The transposon, Tn*5luxAB* contains a promoterless luciferase gene fusion adjacent to the left end of the transposon. The left IS*50* was truncated from the outer limit so that, when inserted into its target, a promoter from the host can express the luciferase genes and produce bioluminescence proportional to the strength of the promoter. By this mutagenesis, mutants defective in siderophore synthesis were generated and characterized. The work has culminated in the discovery of three new unrecognized siderophores, and the siderophore production has been studied from a dynamic point of view in order

to aid in understanding the mechanisms of low-affinity and high-affinity iron uptake systems.

2. MATERIALS AND METHODS

2.1. Bacterial strains and maintenance of strains

The capsule-negative parental strain UW (strain OP; ATCC 13705) and the pyoverdin-minus strain UA1 (Page & Huyer, 1984) of *A. vinelandii* were maintained at room temperature, on iron-sufficient Burk's medium (25 μM Fe) which contained Burk's buffer (0.8 g/l K_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7\ H_2O$, 78 mg/l $CaSO_4$, 0.25 mg/l Na_2MoO_4 pH 7.2) plus 1 % glucose, 1.1 g/L ammonium acetate as nitrogen source, 25 μM ferric citrate as iron source, and 1.8 % agar for the plate medium. On the other hand, iron-deficient Burk's medium (0 μM Fe or 1 μM Fe) contained 0 μM or 1 μM ferric citrate. Iron-deficient (0 μM Fe) Burk's medium normally contained about 0.5 μM iron contamination from the constituents of the medium. For growth experiments in liquid culture, agar was excluded from the medium and the iron-deficient medium usually had either no added iron or 1 μM ferric citrate in order to promote iron-deficient growth (Page & von Tigerstrom, 1988). The inoculum was added at 1 % of the culture volume and that was pregrown at 30 °C incubator for 2-3 days on slants of iron-sufficient Burk's medium (25 μM Fe).

Escherichia coli strain S17-1 (*294 recA, Tp^r, RP4, 2-Tc::Mu-Km::Tn7, pro, res, mod*; Simon *et al.*, 1983; Simon, 1984) containing the suicide plasmid pTn*5luxAB* (*Km^r, Ap^r*) (Berg *et al.*, 1988) and the information regarding the plasmid were obtained from Dr. A.A. Szalay (Plant Molecular Genetics, Faculty of Agriculture, University of Alberta), and *E. coli* strain HB101 (*ampE44, hsd20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1*) containing the helper plasmid pRK2013 (Km^r) (Figurski & Helinski, 1979) was obtained from Dr. L. Frost (Department of Microbiology, University of Alberta). Both strains were grown at 37 °C in TYE medium (15 g/l Tryptone, 10 g/l yeast extract, 5 g/l NaCl, and 1.8 % Agar (for solid medium only) with 50 μg/ml kanamycin for pRK2013, and 50 μg/ml kanamycin plus 40 μg/ml ampicillin for pTn*5luxAB*. Both strains were maintained at slants of TYE with the appropriate antibiotic at room temperature as well as frozen at -70 °C. A loopful of *E. coli* inoculum was used for 20 ml culture volume.

2.2. Tn*5luxAB*-Mutagenesis by conjugation and selection of mutants

Although bi-parental matings worked well later, in earlier experiments tri-parental matings were carried out. Exponentially growing cultures of *A. vinelandii* strain UW and *E. coli* strains pTn*5luxAB*/S17.1 and pRK2013/HB101 (tri-parental mating only) were mated *en masse* in approximately equal volumes. Normally 10 ml of culture from each strain were used in each conjugation experiment. After washing the cultures in Burk's buffer three times, they were concentrated in about 1 ml volume before incubation for mating. Matings were carried out on iron-sufficient Burk's medium plates (25 μM Fe) at 30 °C without using a filter. After 3-4 days the culture was scraped from the plates and resuspended in 1-3 ml of Burk's buffer before spread-plating onto iron-sufficient Burk's medium plates (25 μM Fe) containing 10 μg/ml kanamycin for selecting transconjugants. The Burk's medium did not allow the auxotrophic *E. coli* strains to grow. Isolated colonies were restreaked for purification. In biparental matings *E. coli* strain pRK2013 was omitted.

2.3. Detection of bioluminescence

The mutants obtained after the introduction of pTn*5luxAB* were spot-inoculated (about 5-7 mm diameter) on sectioned plates in duplicate for detection of iron-repressible bioluminescence. Sectioned plates contained Burk's medium with 1 μM and 300 μM ferric citrate in each section respectively. The sectioned plates were incubated at room temperature with 10 μl of n-decanal spread on the lid of the petri dish, with the dish placed agar-side down on a Kodak XAR-5 X-ray film for 15 min. Bioluminescence exposed to the X-ray film was detected after processing the film.

2.4. DNA extraction and transformation of *A. vinelandii*

Plasmid DNA, pTn*5luxAB* was isolated from *E. coli* by the method of Robson *et al.* (1984). Preparation of genomic DNA from *A. vinelandii* was extracted according to the modified protocol by Wilson (1990). Transformation and competence development conditions for *A. vinelandii* were earlier described by Page & von Tigerstrom (1979). However, the 20 h-incubation time for

enrichment of the phenotype was omitted, and the mixture was directly plated out onto 10 μg/ml kanamycin containing iron-sufficient (25 μM Fe) Burk's medium plates because kanamycin did not have an instant bactericidal effect probably due to the differences in the resistance mechanisms.

2.5. Growth experiments

For growth experiments the inocula were pregrown overnight in liquid cultures in iron-deficient (1 μM Fe) Burk's medium at 30 °C water bath shaker incubators at 225 rpm in order to prevent iron-carry over from the inocula. Strains were normally grown in 100 ml media in 500 ml flasks. However, some variations also occurred in growth temperature and agitation rates of the cultures (see Results). The growth was usually monitored by taking appropriate amount of samples for turbidity measurements at time intervals, often 1-2 hours or longer. Turbidity of the cultures were determined at 620 nm using a Novaspec spectrophotometer. Distilled water was used as a blank because it had the same absorbance as the uninoculated medium. However, in some cases growth was not monitored against time spectrophotometrically but the final biomass was used for analyses after one day growth. Some other variations are as follows.

Growth experiments with EDDHA (ethylenediamine-*N,N'*-bis 2-hydroxyphenyl acetic acid) included this synthetic iron chelator at 50 μg/ml concentration.

Nitrogen-fixing growth was carried out under high aeration at 300 rpm, 28 °C in 100 ml media/500 ml Erlenmeyer baffle flasks (each flask contained four radial indentations in its base).

A growth experiment with 2,3 dihydroxybenzoic acid (2,3-DHBA) was performed in order to see whether 2,3-DHBA can aid iron uptake. A 10-fold concentrated solution of 2,3-DHBA was filter sterilized (0.45 μm, Millipore) and added aseptically to at 500 μM final concentrations into 20 ml iron-sufficient (25 μM Fe) and deficient (1 μM Fe) Burk's media in 50 ml flasks.

Growth experiments with increasing iron-concentrations were performed in order to see the relationship between siderophore production and soluble iron in the solution. A range of ferric citrate concentrations of 0, 1, 2, 5, 10, 15, 20, 25, 50,

100, 200, 300, 500, and 750 μM was added into iron-deficient (0 μM Fe) Burk's medium. The cultures were incubated at 28 °C, 225 rpm for 1 day. A parallel series of uninoculated media was incubated as a control for soluble iron determinations.

Growth experiments with iron-containing minerals were performed in order to test the iron-capturing ability of the siderophores through dialysis bags. Crushed minerals (50 mg, about 200 mesh) were included in duplicate 1 cm diameter, 10,000-14,000 MW cut-off dialysis tubing sacs containing 2 ml of iron-deficient (0 μM Fe) Burk's medium. The sealed bags were sterilized in 100 ml of the same medium. One series of the duplicates was used as controls for freely exchangeable iron (Page, 1987). They were incubated under same conditions 1 day before but without inocula. The dialysis bags were removed aseptically from the flasks. Thus, these flasks contained freely exchangeable iron diffused out through the dialysis membrane during 1-day incubation. They were simultaneously incubated with the other series of flasks, containing the mineral iron in the dialysis bags and the freely exchangeable iron in the solution. The growth experiment was carried out at 28 °C, 225 rpm.

2.6. Extraction and visualization of the iron-regulated outer membrane proteins (IROMPs) of *A. vinelandii*

A. vinelandii strains were grown with iron-deficient (1 μM Fe) and iron-sufficient (50 μM Fe) Burk's media. After harvesting the cells by centrifugation, they were broken by sonication (Page & Huyer, 1984). Outer membrane proteins were extracted with sarcosyl treatment (Filip *et al.*, 1973; Page & Huyer, 1984), and 20 μg protein were loaded per lane. They migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system as described Page &von Tigerstrom (1982). The protein bands were visualized with the silver staining method as described by Wray *et al.* (1981), and the profiles of the mutants were compared to that of the parental strain UW.

2.7. Detection of siderophores and chemical assays for quantitation

Siderophores were detected in acidified (HCl, pH 1.8) supernatants by spectrophotometric scanning from 500 nm to 200 nm, and measuring the absorption peaks at 310 nm for catecholate-type siderophores and 380 nm for azotobactin (Page & Huyer, 1984) by means of a Hitachi U-2000 spectrophotometer.

The hydroxamate group of azotobactin was quantitated by the Csaky assay (1948). However, the hydrolysis step was carried out either by autoclaving (1 h, 121 ^{o}C, 1.1 Kg cm^{-1} pressure) or by 6 h boiling. A standard curve was prepared by using a series of dilutions of hydroxylamine hydrochloride.

Catecholate siderophores were quantitated according to the Arnow 's method (1937) modified by Barnum (1977). Concentrations of catecholate siderophores were determined according to a standard curve prepared from a series of dilutions of authentic catechol.

Supernatants were normally extracted three times with an equal volume of ethyl acetate unless otherwise stated. To visualize ethyl acetate extracted fractions, thin layer chromatography (TLC) was performed on silica gel G (Brinkmann), using benzene: acetic acid: water (125:72:3, v/v) solvent system, and the compounds were detected by spraying with 2,2'-bipyridyl-ferric chloride (Krebs *et al.*, 1969).

Non-specific determination of siderophores was also carried out using the chrome azurol S (CAS) plate and shuttle colorimetric tube assays (Schwyn & Neilands, 1987). In both forms of the CAS assay, siderophores are detected independent of their structure. In the CAS plate assay, strains were inoculated as spots on CAS agar plates prepared according to Schwyn & Neilands (1987) except that MM9 salts were replaced with Burk's medium and casamino acids were omitted. Siderophore production was determined by the formation of orange halos around the site of inoculation. In the CAS-shuttle assay, 0.5 ml of CAS-shuttle solution was mixed with 0.5 ml of supernatant and the absorbance at 630 nm was measured over time in a Hitachi U-2000 spectrophotometer immediately. Uninoculated medium was used as a blank. The iron exchange from iron-dye complex to the siderophores was accompanied by a decolorization to a pink or

orange colour and a negative slope in absorbance. Because this assay reacts with all siderophores, it was used for the determination of the total siderophore activity (TSA) in a culture fluid. When samples of culture fluids were extracted with ethyl acetate for use in the CAS-shuttle assay, the ethyl acetate fraction and the aqueous fraction were air-dried overnight to remove ethyl acetate, and ethanol if the ethyl acetate fraction was dissolved in ethanol, since both compounds interfered with the assay. The pH of the solution was always adjusted to about 6.0 prior to the assay. Ten-fold concentrated uninoculated medium did not cause any interference in the CAS-shuttle assay.

2.8. 2,2'-Bipyridyl assay for iron determination

Iron determination was done using cells from 75 ml of culture, harvested by centrifugation and washed in 10 mM sodium citrate, pH 7.2 to remove cell-bound iron. The cell pellet was suspended in 2.5 ml 7 % (w/v) perchloric acid, extracted overnight at room temperature, then heated at 80 °C 4 h before iron quantification. Iron was quantified from the cell-bound fractions that were removed with sodium citrate, from the filtered (0.45 μM, Millipore) blank media, from the filtered supernatants as well as from the intracellular fraction extracted with perchloric acid by 2,2'-bipyridyl assay (Page & Huyer, 1984). Basically, 1 ml sample in 0.35 N HCl was mixed with 0.5 ml of 2 % 2,2'-bipyridyl (in 95 % ethanol) plus 0.1 ml of 0.1 M ascorbic acid. The absorbance of the red colour formed was measured at 530 nm after 10 min. incubation. However, in some cases ascorbate was replaced with other compounds in order to determine their reducing activity (see Results section). All glassware for iron determination was acid-washed (Collinson *et al.*, 1987).

2.9. Other assays.

Cell protein was determined according to Lowry *et al.* (1951) as described by Page & Huyer (1984). Bovine serum albumin was used for preparing the standard curve.

The ninhydrin reaction was determined by autoclaving the samples with 0.25 % ninhydrin at liquid cycle (121 °C, 1.1 kg cm^{-1} pressure). An amino acid putrescine (0.1 %) was used as a positive control.

Citric acid analysis was performed using an enzyme reagent kit purchased from Boehringer (cat. no. 139 076). The principal of the assay as follows. In the assay, citrate is converted to oxaloacetate (OAA) and acetate by the enzyme citrate lyase. In the presence of the enzymes malate dehydrogenase and L-lactate dehydrogenase, OAA and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, with reduced nicotinamide adenine dinucleotide (NADH). The amount of NADH oxidased in the latter two reactions is proportional to the amount of citrate, and it is determined by measuring the absorbance at 340 nm. Samples were 10-fold concentrated by freeze-drying prior to citrate analysis.

Ascorbic acid analysis was done using an enzyme reagent kit purchased from Boehringer (cat. no. 409 677). The principal of the assay as follows. Some reducing agents including L-ascorbic acid reduce the tetrazolium salt MTT [3-(4,5-dimethylthiazoyl-2)-2,5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinium methyl sulfate) at pH 3.5 to an MTT-formazan. L-Ascorbate in a sample blank is specifically determined by the oxidative reaction of ascorbate oxidase. L-Ascorbate is quantified taking the absorbance difference of the sample and the sample blank. The MTT-formazan is the measured parameter, and its absorbance at 578 nm is determined

Glucose determination was carried out using a glucose oxidase enzyme reagent kit purchased from Sigma (Procedure no. 315). The principle of the enzyme assay as follows. The enzyme glucose oxidase breaks down glucose into gluconic acid and H_2O_2 in the presence of O_2. The H_2O_2 formed reacts with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye in the presence of peroxidase with an absorbance peak at 505 nm. The intensity of the colour is directly proportional to the concentration of glucose in the solution.

2.10. Gel exclusion column chromatography

Column chromatography was carried out by using Bio-Gel P-2, which has an exclusion limit of 1,800 Daltons. The size of the column was 1 cm diameter by

32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column was 0.2 ml/min. Fractions were analyzed by using CAS-shuttle assay. To prevent the interferences with CAS-shuttle solution, Milli-Q water (distilled and deionized water) was used as the solvent. This also helped purify the siderophores through the column.

3. RESULTS

3.1. Tn*5luxAB*-Mutagenesis of *Azotobacter vinelandii*

The plasmid, pTn*5luxAB* does not have transfer functions and therefore it would be mobilized by a helper plasmid in interspecies matings. However, conjugation experiments with traditional procedures were unsuccessful (Selveraj & Iyer, 1983). Repeated transformation experiments were also unsuccessful using the purified plasmid DNA. However, several transconjugant colonies appeared on iron-sufficient (25 μM Fe) Burk's medium containing 10 μg/ml kanamycin after 2-3 days of incubation during triparental matings. The transconjugants were not only kanamycin-resistant but also some of them produced bioluminescence when exposed to n-decanal, substrate for the enzyme luciferase. This indicated that pTn*5luxAB* had been transferred into the *Azotobacter* cells. Normally several days were required in order to isolate large numbers of transconjugants. It was also later understood that biparental matings were sufficient for the transfer of the plasmid. In short term matings usually no transformants were obtained. Therefore, the process by which the plasmid was transferred did not appear to be a conjugation in the traditional sense of the word because of the lengthy duration.

The transfer of pTn*5luxAB* into *A. vinelandii* was studied with several different experiments using kanamycin resistance selection. No natural kanamycin resistant colonies of *A. vinelandii* were observed. Using a large number of cells, in one case 2-4 transconjugants per plate were obtained as early as 15 h after mating began. To test if the process was transformation or conjugation a large number of competent *A. vinelandii* cells were incubated with and without 500 μg/ml DNAse. The experiment was repeated with non-competent cells. Normally, *Azotobacter* cells were made competent for transformation by exogenous DNA by growing overnight in iron-deficient (0 μM Fe) Burk's medium. Whereas the iron-sufficient cells were non-competent. Similar data were obtained in the presence and absence of DNAse. After 15 h mating, 2 transconjugants per plate without DNAse and 4 transconjugants per plate with DNAse were obtained with the iron-deficient cells of *A. vinelandii* that were competent. After 24 h mating, competent cells with and without DNAse gave 10 and 8 colonies per plate, respectively, while non-

competent cells with and without DNAse gave 9 and 7 colonies per plate. After 48 h of mating, about 100 colonies were observed in all cases. This was the most efficient experiment in which the highest number of transconjugants were obtained. Normally, fewer, often less than 10, transconjugants were obtained. Essentially, no practical differences were found in the observed number of recombinants between the competent and non-competent cells as well as between the conjugation mixtures incubated with and without DNAse. Since the proc ss was not DNAse sensitive, it must be a form of conjugation. When the Tn*5*-construct in the original host strain *E. coli* S17.1, the cells grew on 50 μg/ml of kanamycin while the transconjugants appeared to have resistance to optimum 10 μg/ml kanamycin. However, the frequency of the transconjugant production was not determined accurately because many of the transconjugants appeared to be siblings since they were able to replicate during the lengthy incubation period. However, normally each conjugation mixture contained around 5×10^8 viable cells/ml for each strain, and once they were concentrated 10-fold for mating, the values became 5×10^9 cells/ml per strain. Thus, for 5-10 transconjugants, one could predict a transconjugation frequency of $0.5\text{-}1.0 \times 10^{-9}$ transconjugants per donor or recipient cell for 24 h incubated mating mixture.

With several matings, over 500 kanamycin-resistant mutants of *A. vinelandii* were obtained. Since pTn*5luxAB* is a suicide plasmid, i.e. not maintained stably in the host *A. vinelandii,* kanamycin-resistant mutants were assumed to be the result of transposition of the transposon into the genome of the organism. The mutants were screened for their ability to produce iron-repressible lux activity. Four groups of mutants were observed: the first group contained many isolates, and did not emit bioluminescence at all; the second group contained very few isolates, and emitted bioluminescence strongly and constitutively; the third group contained many isolates, and emitted iron-repressible bioluminescence moderately; the fourth group contained several isolates, and emitted bioluminescence strongly iron-repressible. Apparently each group also included some siblings which arose during the lengthy incubation period. Furthermore, stable maintenance of the mutants for a long time, even in the absence of the antibiotic, proved that the transposon was stably inserted in the chromosome.

The mutants were also tested for their ability to produce orange haloes on CAS plates in order to detect siderophore defective, over-producing or transport defective mutants as mentioned by Schwyn & Neilands (1987). However, very little difference was found among the mutants with respect to the size of halo and hence the level of total siderophore production.

Because the CAS plate assay was not accurate for quantifying the total siderophore production, the mutants were grown in iron-deficient (0 and 1 μM ferric citrate) and iron-sufficient (10 and 50 μM ferric citrate) Burk's media and the corresponding supernatants were examined for the production of catecholate siderophores (A_{310}/protein) and azotobactin (A_{380}/protein) by measuring the absorbance in a spectrophotometer. The majority of the mutants appeared to produce both siderophores and they had approximately the same absorbances with the parental strain UW. Spectrophotometric scanning of strain UW normally gave peaks at 310 nm and 380 nm corresponding to catecholate-type and azotobactin siderophores, respectively (Fig. 4). However, one mutant strain F196 (Fig. 4) and two other mutants, possible siblings, strains F124 and F174 apparently did not form catecholates but did produce azotobactin (A_{380}/protein) to a level two and half times that of the parent strain. These three strains produced strongly iron-repressible bioluminescence. Another strain D27, did not produce azotobactin (Fig. 4) and did not produce bioluminescence at all. Furthermore, analysis of IROMP profiles of these mutants did not show any difference with the parental strain in SDS-PAGE (data not shown).

Catecholate production by strain F196 was tested by adding exogenous 2,3-DHBA. Strain F196 was grown with 500 μM 2,3-DHBA in iron-deficient (0 μM Fe) Burk's medium. The supernatant was acidified and extracted with ethyl acetate. All the catecholates were extracted into the ethyl acetate fraction, and thin layer chromatography showed that this was 2,3-DHBA exclusively. It was concluded that strain F196 cannot utilize exogenous 2,3-DHBA as a precursor to synthesize catecholate siderophores.

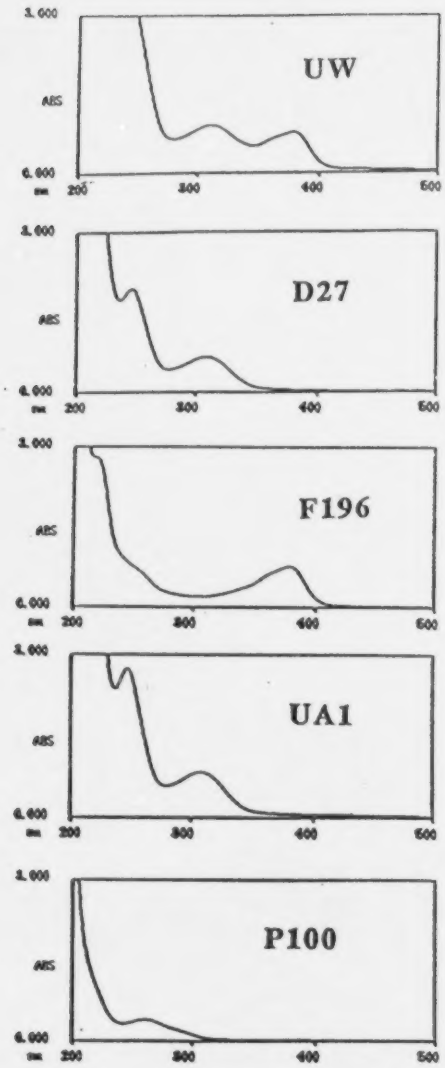


Figure-4. Spectrophotometric scans of acidified culture supernatants of the strains of *Azotobacter vinelandii* (Peaks A_{310}= catecholates; A_{380}= azotobactin).

3.2. Generation of strain P100 that is defective in known catecholate and hydroxamate siderophores

Strain UA1, another azotobactin-negative strain derived from strain UW, had been obtained by spontaneous mutation (Page & Huyer, 1984). The catecholate peak at A_{310} was the only peak observed when the acidified iron-deficient culture supernatant of strain UA1 was examined (Fig. 4). This deficiency of the pyoverdin-type siderophore made it a target for this study in order to obtain a mutant defective for the known siderophores. Although strain D27 had the same phenotype, transformation experiments in order to bring two phenotypes in one transformant strain was unsuccessful. This may have been either due to the lack of appropriate selection technique or the exclusion of the second Tn*5luxAB* from the genome. For this reason, strain UA1 was transformed with the DNA from strain F196. As predicted from the homologous nature of recombination and selective pressure of the bactericidal effect of kanamycin, almost all the transformants appeared to be phenotypically identical (whitish colony colour, small size, no fluorescent yellow-green pigment production). The acidified (as well as non-acidified) iron-deficient culture supernatants of the transformants lacked any peaks at A_{310} and A_{380}. These strains also emitted iron-repressible bioluminescence. This suggested that the insertional mutagenesis of strain UA1 with the Tn*5luxAB* construct happened possibly in a site-directed manner, and the two mutant phenotypes, the catecholate deficiency from F196 and the pyoverdin deficiency from UA1 had converged in one single strain. One colony was picked and designated as strain P100 (Fig. 4).

3.3. Growth of siderophore-deficient mutants and the parental strain UW in iron-sufficient and iron-deficient media

The siderophore-defective mutant strains D27, F196, UA1, P100 and the parental strain UW of *A. vinelandii* were grown in iron-sufficient (25 μM Fe) and iron-deficient (1 μM Fe) Burk's media. Fig. 5 shows growth curves of iron-sufficient cultures, and one can see two different exponential growth phases. This is because *A. vinelandii* is known to have a diauxic growth when grown with citric acid cycle intermediates (George *et al.*, 1985; McKenney & Melton, 1986), such as

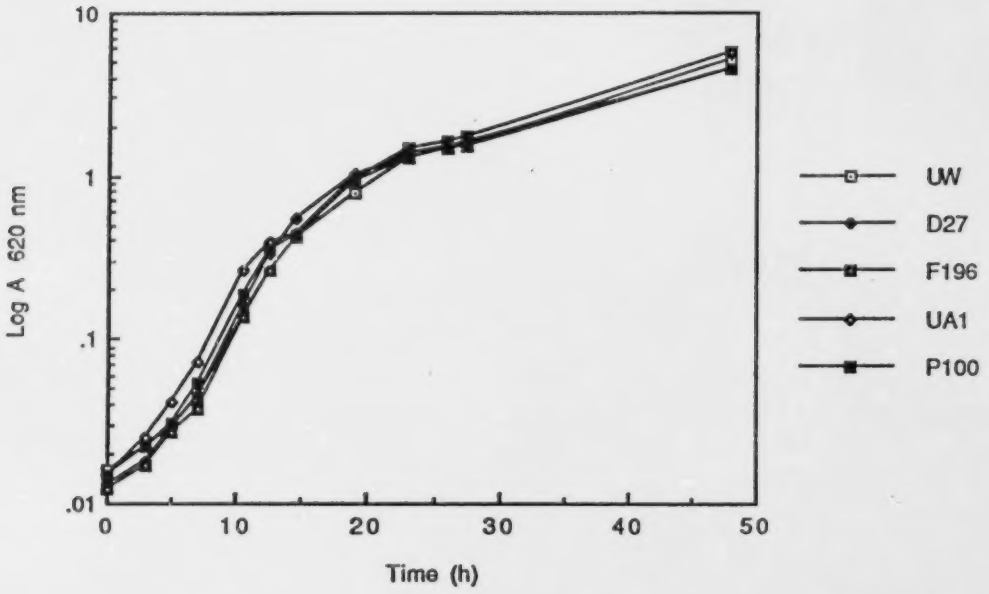


Figure-5. Growth curves of the strains of *A. vinelandii* grown in iron-sufficient (25 μM Fe) Burk's medium (all strains were grown at 30 °C, 225 rpm).

acetate, from the ammonium acetate component of the medium which is preferred over glucose. Thus, the first exponential phase occurred due to the consumption of acetate followed by the second exponential phase in which glucose was consumed. However, no practical variation was found among the mutants and the parental strain UW when the cultures were grown in iron-sufficient media. Nevertheless, some differences appeared among them during the second exponential phase when grown in iron-deficient media (Fig. 6). All mutants grew slower and with less turbidity than that of the parental strain UW in the second exponential phase. Strain P100 was the slowest and yielded less biomass than the others, indicating that iron-deficiency had an effect on its growth. Nevertheless, this did not have much effect on the overall growth pattern of the mutants. This gave the evidence that there may be another siderophore or means of getting iron into the cell other than these two known major siderophores, azotobactin and 2,3-DHBA derived-catecholates.

The cell protein content of the mutants, grown with sufficient and limited concentrations of iron, were compared, and it was found that all strains lowered their protein content when iron was deficient in the medium (Table-1). However, the parental strain UW was less affected than the mutants. Strains P100 and F196 were most affected.

3.4. Siderophore production of strains

Hydroxamate and catecholate siderophores were quantified from iron-deficient supernatants of the parental strain and siderophore-defective mutants (Table-2). Azotobactin-defective strains, D27 and UA1 did not produce the siderophore, azotobactin. Both strains produced approximately the same amount of catecholate as the parent strain. On the other hand, the catecholate-defective strain F196 (and its siblings, data not shown) overproduced azotobactin as much as 2.5 times that of the parent strain. The low level of catecholate detected in the supernatants of strains F196 and P100 was not extracted into ethyl acetate and was not detected by thin layer chromatography. On the other hand, the yellowish colour of azotobactin from strain F196 was retained at the pH of the assay, possibly it had a slight effect on the readings since addition of NaOH did not change the yellowish colour.

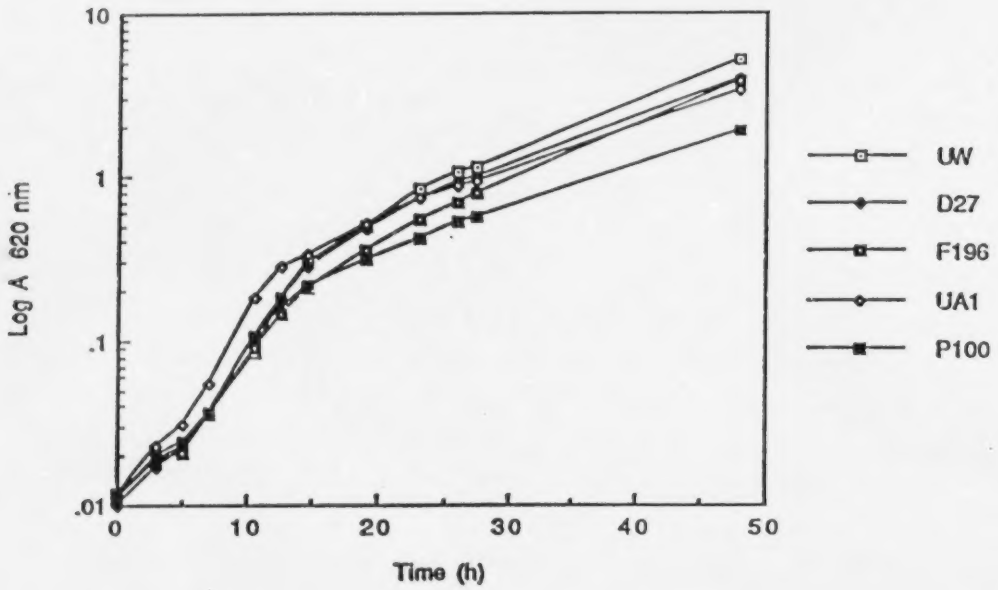


Figure-6. Growth curves of the strains of *A. vinelandii* grown in iron-deficient (1 μM Fe) Burk's medium (all strains were grown at 30 °C, 225 rpm).

Table-1. Comparison of cell protein content (μg/ml) of strains grown in iron-sufficient (25 μM Fe) and iron-deficient (1 μM Fe) Burk's medium.

Time incubated (hours)	Iron-sufficient grown strains				
	UW	D27	F196	UA1	P100
27.5	736	607	639	863	617
48	1056	1011	992	1085	881

Time incubated (hours)	Iron-deficient grown strains*				
	UW	D27	F196	UA1	P100
27.5	251 (34.1)	156 (25.6)	99 (15.4)	217 (25.1)	106 (17.1)
48	396 (37.5)	313 (30.9)	276 (27.8)	333 (30.7)	261 (29.6)

* % Protein yield achieved as compared to the iron-sufficient cultures of the iron-deficient media are given in brackets.

Table-2. Characterization of siderophore-defective strains*

Strains	Cell protein (μg/ml)	Iron-repressible bioluminescence	Hydroxamate A_{380}†	Hydroxamate (μg/mg protein)‡	Catecholate A_{310}†	Catecholate (mM/mg protein)x	Cellular iron (ng/mg protein)
UW	389	N/A	2.83	1.63 (1.61)	3.24	1.48	210
D27	310	No	0	0 (0)	2.75	1.38	202
F196	348	Yes	6.96	4.06 (4.26)	0	0.04	209
UA1	402	N/A	0	0 (0)	3.31	1.59	223
P100	229	Yes	0	0 (0)	0	0.03	197

* All strains were grown for 24 h at 30 °C at 225 rpm in iron-deficient (1 μM Fe) Burk's medium. All values are means of at least duplicates from a single flask.

† Absorption of acidified supernatants per mg total cell protein.

‡ Hydrolysis was performed for 1 h in the autoclave or for 6 h at 100 °C (value in brackets). Units of μg/ml hydroxylamine equivalents.

x Units of mM/ml authentic catechol equivalents.

N/A: not applicable

The siderophore-defective strains, except strain P100 grew almost as well and accumulated approximately the same amount of iron from the medium as did the parent strain (Table-2). However, strain P100 despite its efficient growth under iron-sufficient conditions, in iron-deficient media its protein biomass content remained at 59 % of that obtained with the parental strain without having much effect on the deposited iron. Basically all strains appeared to internalize similar amounts of iron when grown in iron-deficient media.

3.5. Effect of EDDHA on growth of strains

The synthetic iron-chelator EDDHA binds iron strongly. This compound was included at 50 μg/ml concentration in iron-sufficient (25 μM Fe) Burk's medium, and the growth curves of all five strains were followed by turbidity measurements. It was found that the parental strain UW and strains, D27, F196 and UA1 grew efficiently in this medium while the growth rate of strain P100 decreased greatly (Fig. 7). When these strains were grown as inocula in iron-deficient (0 μM Fe) Burk's medium, and inoculated subsequently into iron-sufficient (25 μM Fe) Burk's medium containing EDDHA, the growth of strain P100 ceased while no apparent effect was observed in the other strains (Fig. 8).

The inhibitory effect of EDDHA on the growth of strain P100 was also demonstrated on solid medium. The experiment was designed as a simple, but multi-purpose bioassay, so that not only the inhibitory effect of EDDHA but also the ability of strain P100 to utilize siderophores from other strains by cross-feeding could be measured, i.e., if strain P100 is incubated as spots alone, it will not grow; however, if the other siderophore producing strains were incubated as nearby spots to the spots of strain P100, then strain P100 would grow by utilizing the siderophores secreted into the agar by the other strains. The duration of the lag phase depended on the stored iron content of the inoculum: it was about 5 days if the inoculum was grown in iron-sufficient (25 μM Fe) medium or about 7 days if the inoculum was grown in iron-deficient (1 μM Fe) medium, and eventually, the colony size of strain P100 was able to keep up to the growth of the parent strain in 1 or 2 weeks respectively.

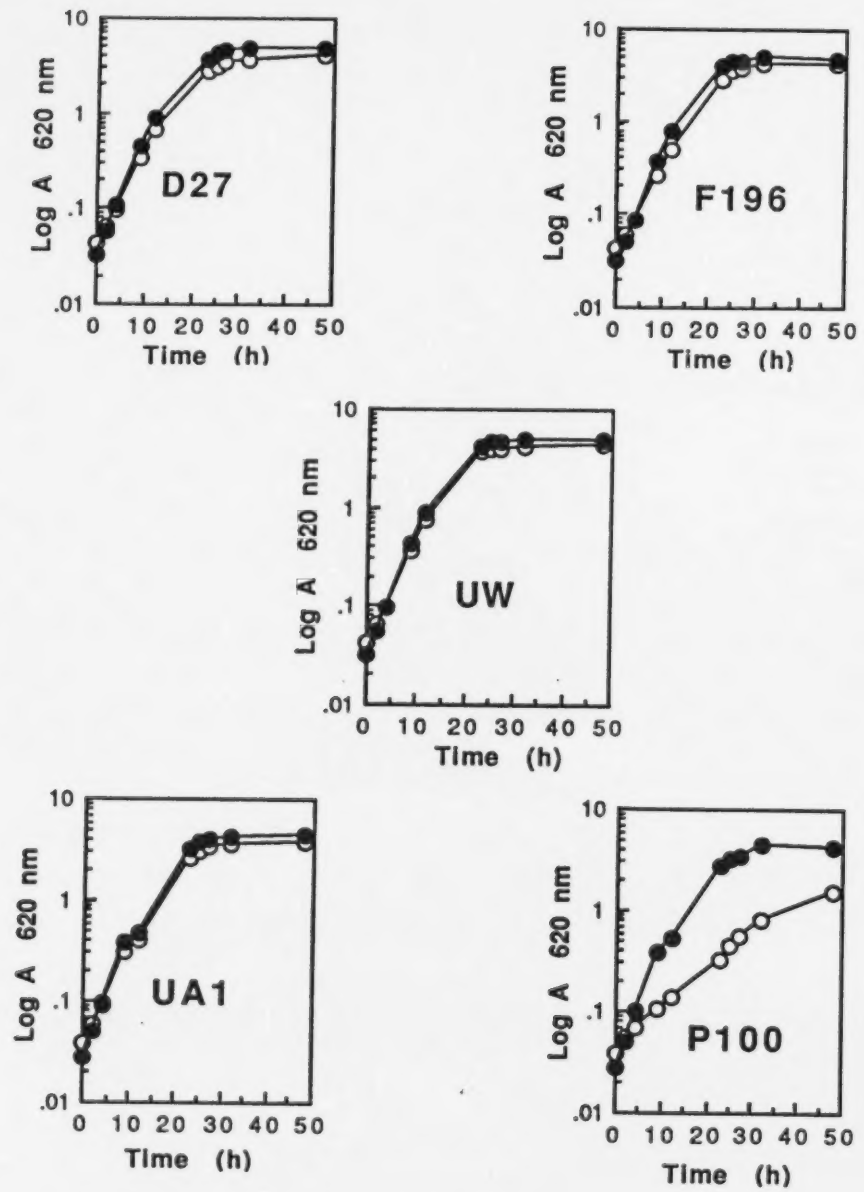


Figure-7. Effect of EDDHA on growth of the siderophore-defective strains and the parental strain UW of *A. vinelandii* with iron-sufficient grown inocula (strains were grown with (○) and without (●) EDDHA (50 μg/ml) in iron-sufficient (25 μM Fe) Burk's medium; the inocula were grown with 25 μM added iron).

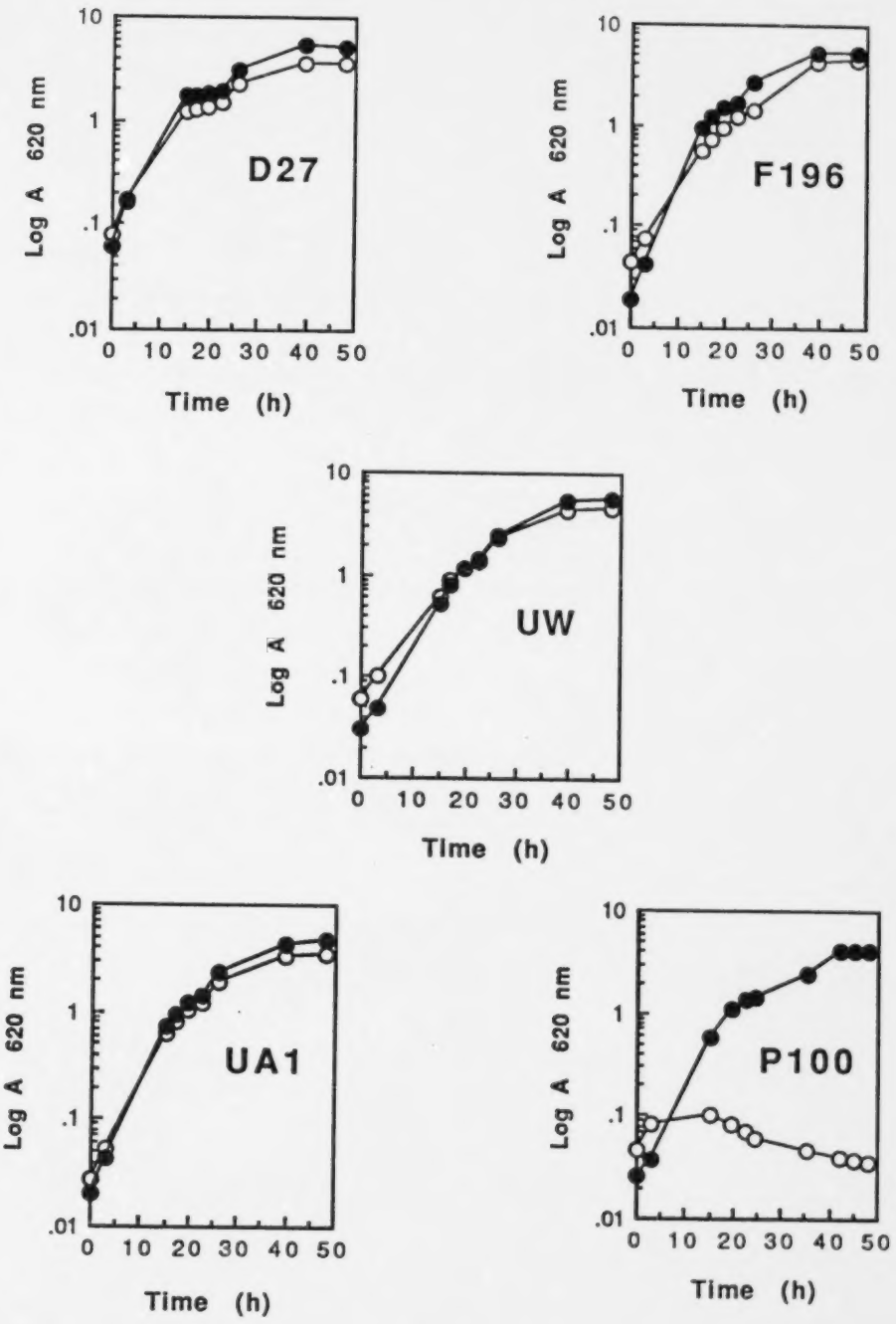


Figure-8. Effect of EDDHA on growth of the siderophore-defective strains and the parental strain UW of *A. vinelandii* with iron-deficient grown inocula (strains were grown with (○) and without (●) EDDHA (50 μg/ml) in iron-sufficient (25 μM Fe) Burk's medium when the inocula were grown with zero added iron).

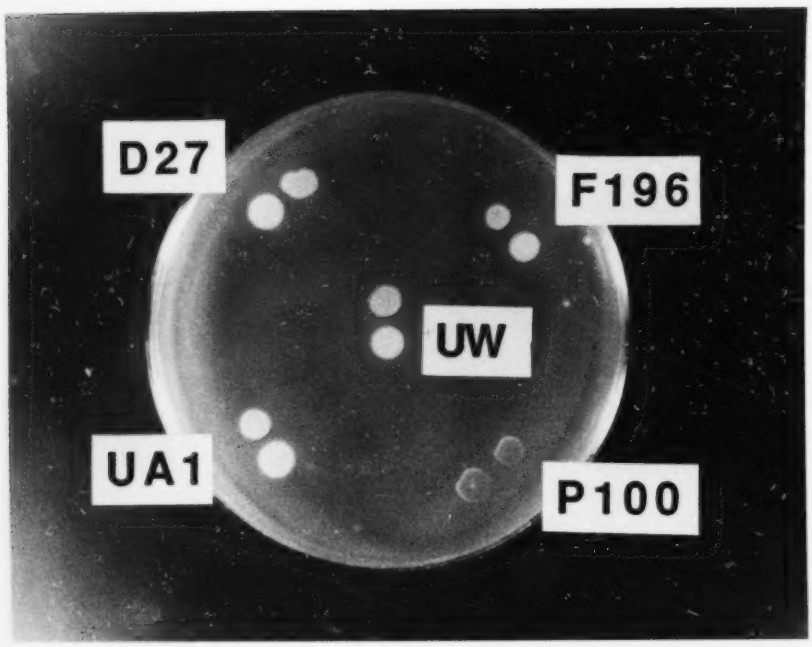


Figure-9. Cross-feeding of strain P100 by the products of other strains. Iron-deficient (0 μM Fe) overnight grown cultures (lower spot of pair) were inoculated along with strain P100 (upper spot of pair). Plate shows supported growth of strain P100 after 8 days of growth at 30 °C.

When no ferric citrate was included in the medium, the growth of strain P100 was dramatically inhibited and no apparent growth was observed in the first 3-4 day of incubation. However, strain P100 was not killed by this treatment but was able to recover after a lag phase (Fig. 9). Cross-feeding of strain P100 with the siderophores from other strains showed that the growth inhibitory effect of EDDHA on strain P100 was reversed by siderophores secreted into the agar medium by the other mutants (Fig. 9). However, the products secreted from the adjacent two spots of strain P100 did not promote the growth of strain P100.

On the other hand, when the agar plates contained 25 μM ferric citrate with EDDHA, there was no effect on the growth of the strains (plate not shown). All strains grew efficiently whether the inocula were pre-grown in iron-deficient (0 μM Fe) Burk's medium or not.

3.6. Testing strain P100 for siderophore production by the CAS assay

Growth of strain P100 on a CAS plate resulted in an orange halo around the site of inoculation after 24 h incubation (Fig. 10). This latest finding was unexpected, since previous results had shown that strain P100 was missing all known siderophores. This result could lead one to think that either the organism was producing a compound that was interfering with the CAS assay or was producing a non-conventional siderophore (neither catecholate nor hydroxamate-type) (Schwyn & Neilands, 1987).

The CAS plate results were later confirmed with the CAS-shuttle assay. Supernatants from all strains that had been grown under iron-deficient (1 μM Fe) medium were mixed with the CAS-shuttle solution and the ΔA_{630} values were recorded at 10 sec intervals until decolorization was complete. The comparative results from other strains indicated that strains UW, D27 and UA1 had approximately similar total siderophore activities in the assay (Table-3). Strain, F196 had a value of about 60 % of the parent strain while the value obtained with strain P100 remained at about 30 % of the parent strain. Apparently, only catecholate-producing strains D27 and UA1 as well as the parent strain UW deferrated the CAS-ferric complex rapidly, while the catecholate-negative strain

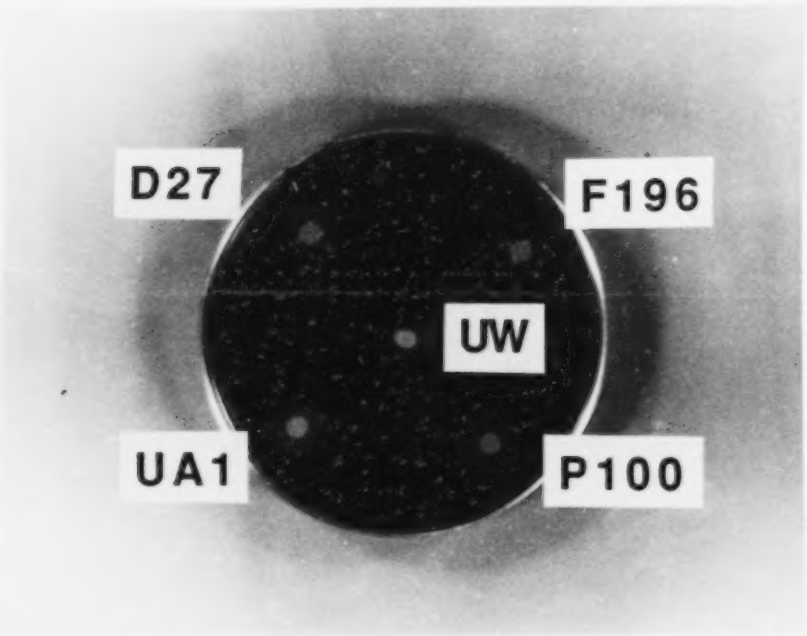


Figure-10. Siderophore production by the strains on a CAS plate. In each spot, 2 µl of overnight-grown cultures were dropped on the plate, and incubated 1 day at 30 °C.

Table-3. Total siderophore activity of strains as estimated with the CAS-shuttle assay*.

Strain	Total siderophore activity		End point (min)
	Units†	Specific activity‡	
UW	22.5	118	1-2
D27	22.1	142	1-2
F196	13.3	75	5-6
UA1	20.5	104	1-2
P100	7.1	61	≥ 15
EDDHA§	9.1	N/A	10-15

* All strains were grown as described in Table-2. CAS-shuttle assay was performed at 37 °C with native supernatants.

† Total siderophore activity, where one unit (U) is $\Delta A_{630\ nm}$ of 0.001 per sec per 0.5 ml supernatant used in this assay.

‡ Total siderophore specific activity, as U per mg cell protein present in the culture fluid volume assayed.

§ EDDHA (50 µg/ml) used from an uninoculated medium.

N/A: Not applicable.

F196 did so relatively slow, and strain P100 was the slowest. Deferration ability of strain P100 was also compared to that of the synthetic iron-chelator, EDDHA. EDDHA (50 μg/ml) apparently deferrated the complex with a slightly higher affinity than that of strain P100. This probably was enough to account for the ability of this concentration of EDDHA to inhibit the growth of strain P100 (Figs. 7, 8 and 9). On the other hand, when these values were normalized with the protein contents of the strains, strain D27 had the highest specific activity, followed by strain UW and strain UA1, having specific activities, 142, 118, and 104 units, respectively. Strain P100 had the lowest specific activity, 61 units, while strain F196 had a somewhat higher value of 75 units. The discrepancy between the specific activities obtained with strains D27 and UA1 suggested that mutational sites in both strains were at different loci although both lacked the azotobactin siderophore.

Furthermore, the supernatant of strain P100 was assayed for the detection of citrate since citrate has been shown to act as a non-conventional siderophore in *Azotobacter salinestris* (Page, 1987; Page & Shivprasad, 1991). Nevertheless, citrate was not detectable even when the 10-fold concentrated supernatant of strain P100 from iron-deficient growth was used.

3.7. Iron-mobilization from natural iron sources by strain P100

The ability of strain P100 to mobilize iron from insoluble iron sources was tested by including the minerals in iron-deficient (0 μM Fe) Burk's medium. The iron containing mineral was included in a dialysis bag, so that the cells would obtain the iron with their siderophores through the membrane but not by direct contact. Siderophores, by definition, should be small enough to penetrate through the dialysis membrane to capture iron and bring it to the cell (Neilands, 1981a). Strains UW and P100 were pregrown in iron-deficient (0 μM Fe) Burk's medium overnight and inoculated with minerals as the iron source.

The results showed that strain P100 efficiently utilized the minerals FeS, vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$], and to a lesser extent Fe_3O_4 (Table-4). The total cell protein content of strain P100 increased and the cells accumulated more iron when grown in the presence of these minerals in the dialysis bags than when strain P100 was grown in iron-deficient (0 μM Fe) Burk's medium containing freely

Table-4. Solubilization of iron from iron-containing minerals included in dialysis bags*.

	UW				P100			
Minerals	Freely exchangeable† iron (ng)	protein (μg)	Cellular‡ iron (ng)	protein (μg)	Freely exchangeable† iron (ng)	protein (μg)	Cellular‡ iron (ng)	μg protein
None	9.6 ± 0.3	64.6 ± 2.4	11.6 ± 1.9	94.0 ± 4.7	6.1 ± 0.1	50.0 ± 10.1	5.9 ± 1.2	53.3 ± 1.4
Fe_3O_4	10.0 ± 0.2	70.4 ± 2.4	112.6 ± 7.4	741.4 ± 32.3	10.6 ± 1.3	50.8 ± 1.2	34.6 ± 2.0	283.4 ± 7.2
Vivianite§	16.4 ± 0.8	77.3 ± 4.0	254.8 ± 1.4	817.3 ± 17.8	16.1 ± 0.6	53.2 ± 3.5	105.0 ± 1.4	504.7 ± 9.8
FeS	22.0 ± 1.4	204.7 ± 5.9	650.8 ± 7.8	933.8 ± 12.5	15.9 ± 0.9	94.9 ± 4.7	192.1 ± 4.8	606.2 ± 11.4

* The data are means ± standard deviations of two or more duplicate.

† Iron mineral contained in the dialysis bag was shaken for 24 h at 28 °C, 225 rpm, the bag was removed, and then inoculum was added and continued to be shaken for another 24 h.

‡ Inoculum was added when iron-containing mineral being incubated.

§ $Fe_3(PO_4)_2 \cdot 8\ H_2O$.

exchangeable iron from the minerals. In the latter set of flasks, the inocula were added after the dialysis bags were removed after 24 h shaking and thus the growth occurred in the presence of the iron diffused out during preincubation period. On the other hand, an increase in growth of strain P100 was not seen with the minerals olivine [$(Mg,Fe)_2SiO_4$], hematite (Fe_2O_3), siderite ($FeCO_3$) or goethite [FeO(OH) in iron-deficient (0 μM Fe) Burk's medium (data not shown), while the parental strain UW grew efficiently in the presence of these minerals (data not shown; Page & Huyer, 1984).

3.8. Nitrogen-fixing growth of strains under vigorous aeration

Under vigorous aeration and nitrogen-fixing conditions the iron demand of the cells increased to meet the higher rate of growth as well as the requirement for the enzyme nitrogenase and respiratory protection of the nitrogenase system, hence the cells were stressed for iron. In all media the doubling time of strain P100 was notably increased in comparison with the other strains (Table-5). Increasing the iron concentration of the medium to 1 μM from 0 μM enhanced the growth rate of all strains by almost the same order. Even a further increase in iron concentration to 25 μM did not improve the growth of strain P100 to that of the parent strain. Furthermore, as a result of higher iron demand under these conditions, even 25 μM iron was not sufficient to repress siderophore production by the strains. In this medium, strains UW, D27 and UA1 produced about 30, 45 and 46 μM catecholate siderophores per mg protein, and strain F196 always released a little azotobactin. These results were later confirmed by concentrating their supernatants 10-fold, including strain P100, and reacting them with the CAS shuttle solution (Table-5).

3.9. Effect of sodium citrate on growth of strains

When all strains were incubated in iron-sufficient (25 μM Fe) Burk's medium including 10 mM sodium citrate, it was observed that all strains failed to grow after about 10 h of incubation (Fig. 11). However, when the pH of the medium was checked it was found to be alkaline (pH 8.26 for UW after 24 h incubation). The medium was normally buffered with 5 mM potassium phosphate buffer (pH 7.2) system. However, this buffering system apparently failed in the presence of 10 mM sodium citrate. When the concentration of sodium citrate was

Table-5. Comparison of the growth of the strains under nitrogen-fixing and at high aeration*.

	Doubling time (h)						
	[Fe]			Iron-sufficient culture†			
Strain	0 μM	1 μM	25 μM	Hydroxamate‡	Catecholate§	CAS (AQ)ƒ	CAS (EA)ƒ
UW	3.1	2.7	3.1	0	30	+	+
D27	4.0	3.0	3.2	0	45	+	+
F196	3.5	2.7	2.9	0.19	0	+	-
UA1	4.0	3.0	3.1	0	46	+	+
P100	4.6	3.4	3.5	0	0	+	-

* Cultures were grown at 28 °C, at 300 rpm, 24 h, in baffle flasks, in Burk's nitrogen-free medium containing 0, 1, and 25 μM ferric citrate.

† Iron-sufficient Burk's medium contained 25 μM ferric citrate.

‡ Hydroxamate was determined as the A_{380} of acidified supernatant per mg total cell protein.

§ μM Catecholate/mg protein was determined according to the Barnum assay (1977).

ƒ CAS assay results using the aqueous phase after ethyl acetate extraction (CAS-AQ) or the ethyl acetate extract (CAS-EA) of 10-fold concentrated iron-sufficient culture supernatant fluids. A positive result was indicated by a decrease in A_{630} of ≥ 5 U (according to Table-3) which continued for at least 120 sec. A negative result was no net decrease in A_{630} over 120 sec.

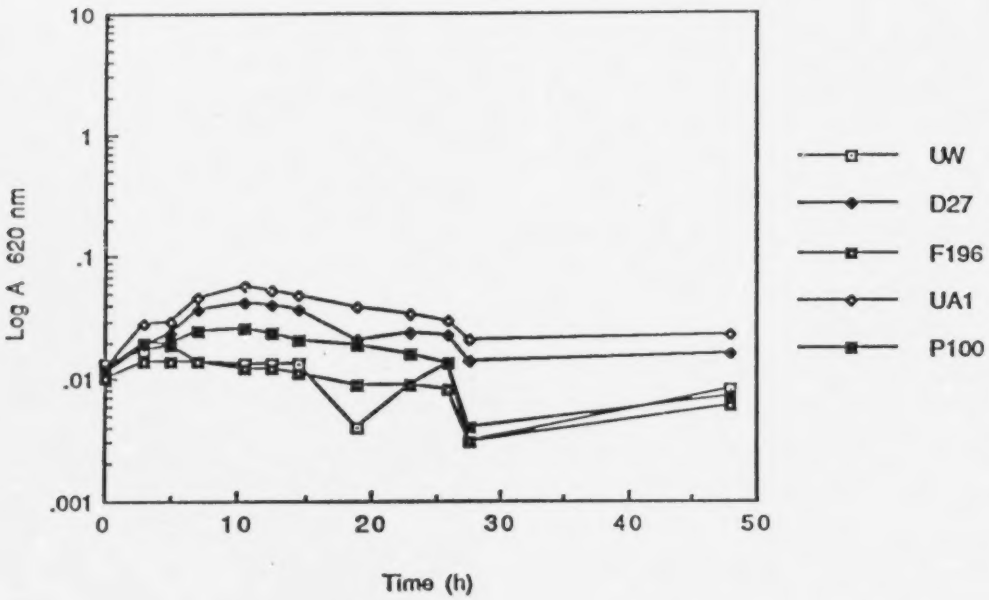


Figure-11. Growth curves of the strains of *A. vinelandii* grown in iron-sufficient (25 μM Fe) Burk's medium with 10 mM sodium citrate (all strains were grown at 30 °C, 225 rpm).

3.5 mM the growth was negligibly affected and the pH remained at 7.31 (data not shown). This was due to the diauxic growth of the organism as explained in section 3.3. Preference of acetate or citrate over glucose resulted in an increase at medium pH. Because acetate and citrate were acid components of the medium, and once they were used, only alkaline components (mainly ammonia) were left that increased the pH. When it reached a certain point, growth ceased. This increase in pH, however, in normal Burk's medium, was overcome later by metabolizing ammonia and glucose, which caused a decrease in the pH. This phenomenon was also observed by monitoring pH after one day and two days of the growth experiment in iron-deficient media in Fig. 6 (Fig. 12). The pH dependent inhibition of growth was also confirmed later by inoculating strain UW in iron-sufficient Burk's medium by increasing the concentration of ammonium acetate two-fold and excluding glucose from the medium; the growth of strain UW ceased after 10 h growth (data not shown). All mutants showed higher pH increases than that of the parent strain after one day-growth. They also were slower than strain UW to decrease the pH of the medium. Here, strain P100 was affected most. Therefore, these pH changes in the medium may also cause slower growth of the mutants. In other words, the siderophore-defective mutants were not as efficient as the parent strain to metabolize glucose in the medium.

3.10. Accumulation of iron and expression of general siderophore activities by strains UW and P100 grown with increasing concentrations of iron

Both strains UW and P100 were grown in Burk's medium with increasing concentrations of iron: 0, 1, 2, 5, 10, 15, 20, 25, 50, 100, 200, 300, 500 and 750 μM, added as ferric citrate. Structurally known siderophores from strain UW were quantified with specific chemical assays, and the CAS-shuttle assay was used as a general assay for detection of siderophores from both strains. Both strains UW and P100 appeared to have a great ability to internalize iron when grown under iron-deficient as well as iron-abundant conditions (Fig. 13). Although strain P100 was defective in known siderophores of strain UW, its ability to store iron was not

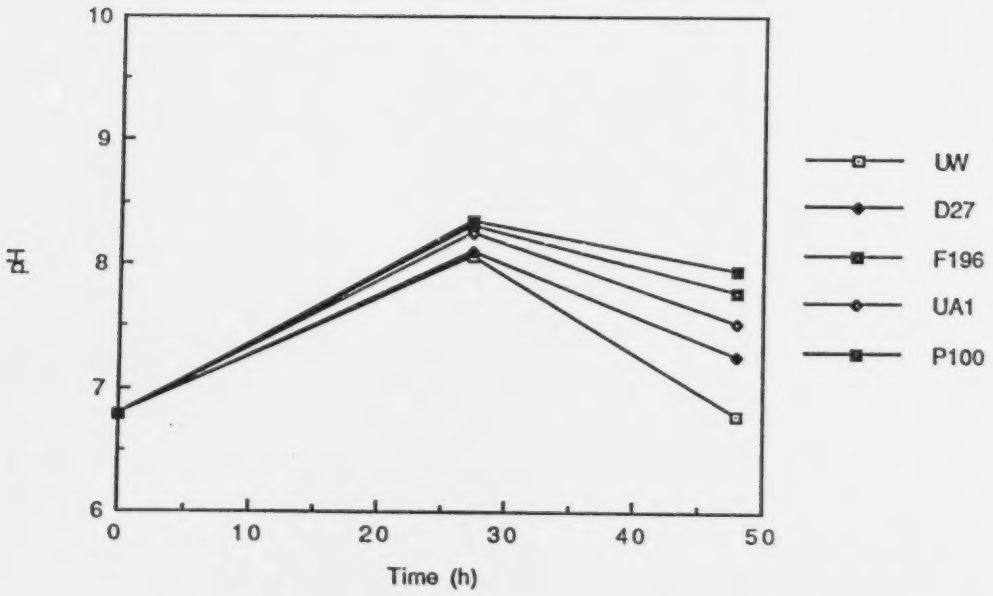


Figure-12. Change of pH during growth of the strains of *A. vinelandii* in iron-deficient (1 μM Fe) Burk's medium (data were taken from Fig. 6).

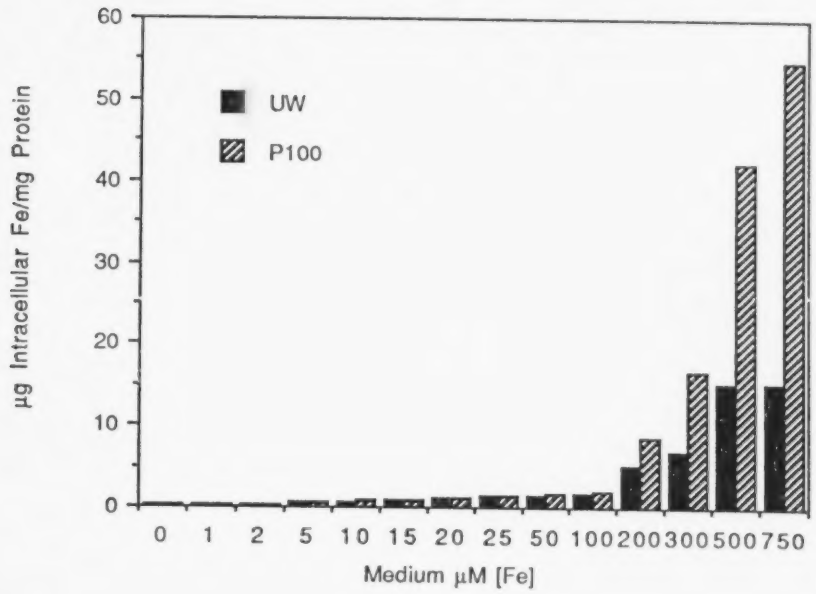


Figure-13. Effect of increasing iron concentrations on intracellular accumulation of iron by strain UW and strain P100. (All cultures were grown 24 h at 28 °C at 225 rpm, and the surface-bound iron was removed with sodium citrate prior to the analysis).

impaired. In fact iron-storage ability of strain P100 was much greater than that of strain UW when the iron concentration of the medium reached 200 μM and greater.

On the other hand, in the case of the cell-bound iron, the picture appeared to be the reverse. Normally when grown in iron-sufficient media *A. vinelandii* accumulates much of the iron in a loosely-bound form outside the cell without internalizing it. This iron can be removed from the cell surface by washing with 10 mM sodium citrate (Page & Huyer, 1984), and this concentration of sodium citrate was shown to inhibit the growth in a pH-dependent manner rather than deferration of the cell surface (see section **3.9**). The surface iron-binding ability of both strains, UW and P100 was tested from the sodium citrate extracted samples. It was found that both strains did not show much difference up to about 100 μM iron in the medium (Fig. 14). However, after 200 μM, the iron-binding ability of the parent strain increased considerably compared to strain P100. However, strain UW was not able to internalize most of this cell-bound iron. Internalization of large amounts of iron during growth with high-concentrations of iron by strain P100 suggested that this mutant strain must have had a mechanism for this process.

Analyses of supernatants of strains P100 and UW with CAS-shuttle solution showed that siderophore activity was present in both supernatants and was repressed gradually with increasing concentrations of iron (about 25 μM for strain UW and about 100 μM for strain P100; Table-6). Moreover, strain P100 had less protein than strain UW under the same growth conditions (Table-6). However, both strains did not increase their protein content greatly above 5 μM iron in the media. Interestingly, above this iron concentration, the parental strain UW ceased or dramatically reduced production of its major siderophores. However, CAS-shuttle activity was still present in culture fluids.

Because of the operation of such a mechanism under high-iron containing media, one could suggest that a low-affinity iron uptake system must be functioning. In the case of strain P100 this process must be over-expressed, so that strain P100 was able to internalize higher amounts of iron than strain UW. This reasoning is also supported by the fact that a charged molecule, like Fe^{3+} will not pass through the membrane freely. Therefore, there must be a mechanism to aid this massive uptake process of iron into the cell. It has been well established that

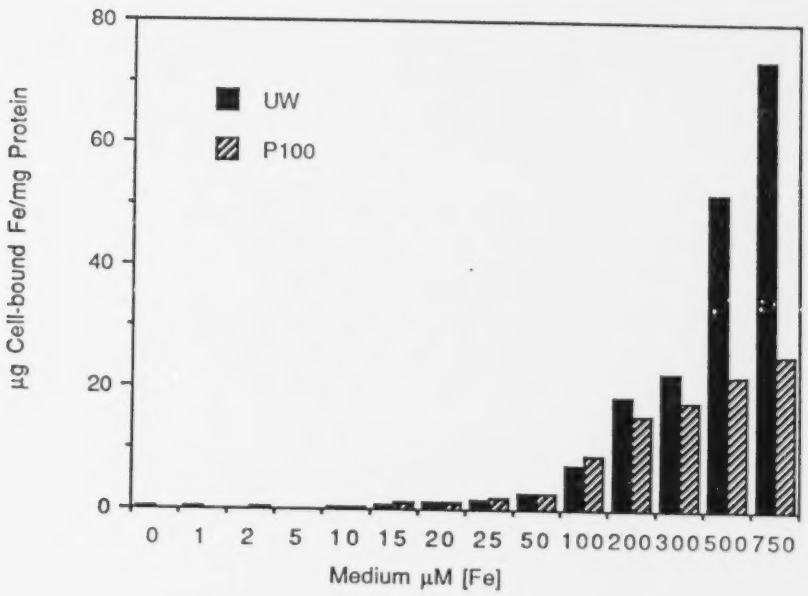


Figure-14. Effect of increasing iron concentrations on surface iron-binding capacity of strain UW and strain P100. (All cultures were grown 24 h at 28 °C at 225 rpm, and the cells were washed with sodium citrate in order to extract surface-bound-iron for analysis).

Table-6. Comparison of siderophore production and siderophore activities of strains UW and P100 with increasing iron concentrations.

Added [Fe] (μM)	Added [Fe] (μg/ml)	soluble† Fe found in Blank (μg/ml)	UW only Catecholateƒ (μM/mg protein)	UW only Hydroxamate§ (μg/mg protein)	Specific TSA‡ UW	Specific TSA‡ P100	Cell Protein (μg/ml) UW	Cell Protein (μg/ml) P100	CAS-shuttle* UW	CAS-shuttle* P100
0	0	0	1201	1.69	262	101	45	38	+	+
1	0.06	0	1102	1.59	230	91	177	84	+	+
2	0.11	0	788	0.64	123	84	232	119	+	+
5	0.28	0	234	0.10	43	41	333	253	+	+
10	0.55	0	39	0	8	23	372	281	+	+
15	0.82	0	23	0	6	16	387	283	+	+
20	1.10	0	21	0	2	9	362	292	+	+
25	1.37	0	19	0	2	7	369	303	+	+
50	2.73	0.03	17	0	0	4	400	303	+	+
100	5.47	0.17	16	0	0	1	409	292	+	+
200	10.9	2.15	17	0	0	0	347	269	+	+
300	16.4	6.07	12	0	0	0	360	283	+	+
500	27.3	23.0	10	0	0	0	343	285	-	+
750	41.0	31.0	4	0	0	0	344	316	-	+

† Soluble iron was determined from the same media incubated under identical conditions without the inocula and filtered through 0.45 μm filter prior to iron determination.

ƒ Catecholate values were determined as units of μM/ml authentic catechol equivalents according to Barnum assay (1977).

§ Hydroxamate values were determined as units of μg/ml hydroxylamine equivalents according to Csaky assay.

‡ Specific total siderophore activity (TSA), where 1 unit (U) is a ΔA_{630} of 0.001 per sec per 0.5 ml culture supernatant fluid used and normalized with cell protein present in the culture fluid.

* CAS-shuttle assay was performed qualitatively by using 10-fold concentrated culture supernatant fluids. A positive result was indicated by a decrease in A_{630} of ≥ 3 U for strain UW and ≥2.4 U for strain P100 and lasted over 60 sec as monitored. A negative result was no net decrease in A_{630} over 60 sec.

siderophores and their receptor proteins are induced under iron-deficient conditions. However, one would also wonder whether this switch from high-iron to low-iron medium induces elements of a high-affinity iron uptake system in an all-on or all-off mode, or whether there is a relative degree of switching mechanism involved in the process. Certainly, the specific assays for detection of siderophores had an apparent limit of detection. If these compounds were present at only low concentration, they would remain undetected although they would be functional. Therefore, one must design some more sensitive techniques in order to demonstrate their presence. For this reason, a feasible, and inexpensive technique was designed. It simply involved concentration of the culture fluids by freeze-drying so that low concentrations of the compounds could be increased in solution. Logically, this method was practical. However, one problem seems in Table-6 was that at increasing iron-concentrations ($\geq$ 50 μM) the soluble iron in the solution would also increase and the yellowish colour of the ferric citrate would interfere with the colorimetric assays used for detection of siderophores. However, from Figs. 13 and 14 one can see that most of the added iron is found either in the cell or bound on the cell and the rest remains insoluble in the solution. Indeed, analysis of the culture supernatants from both strains UW and P100 grown up to 750 μM iron concentrations showed that practically no iron was present in the solutions after 24 h growth (data not shown). However, one could ask the question again if siderophores were involved in the process of acquiring iron from high-iron media, and to what extent. For this reason, the concentrated, filtered, supernatants from both strains UW and P100 were analyzed with CAS shuttle assay. It was found that siderophore activity was present in supernatants of strain UW up to 300 μM iron, and in the case of strain P100 this was up to at least 750 μM iron (Table-6). Perhaps, these results may explain why the mutant strain, P100, can internalize larger amounts of iron than the parent strain. It was apparent that the CAS-shuttle assay was also sensitive to iron concentrations in the solution. Soluble iron in the solution would reverse the CAS-shuttle reaction, i.e. the colour reaction would become denser blue. Therefore, some sort of siderophore activity must be involved in the process.

When the blank Burk's media, without the inocula, containing the same range of iron concentrations were incubated under identical conditions, no soluble iron was found up to 25 μM iron concentrations, while little soluble iron was found in 50 μM and 100 μM iron containing media (Table-6). However, the blank media containing ≥ 200 μM iron contained increasingly larger amounts of soluble iron. Nevertheless, when the bacteria was inoculated, almost all this iron was found largely either in cell bound form as in the case of the parental strain UW or internalized as in the case of the mutant strain P100 (Fig. 13 and Fig. 14). Practically, no soluble iron was found when the filtrates of these cultures were analyzed as mentioned earlier, probably all the remaining iron oxidized to insoluble compounds like ferric hydroxide.

3.11. Siderophore production by strains UW and P100 grown with increasing iron concentrations

It was found that at 10 μM iron and above concentrations, the hydroxamate siderophore of strain UW was no longer detectable (Table-6). Specific total siderophore activity declined when the iron concentrations reached about 20-25 μM (Table-6). Some residual catecholates were detected at iron concentrations ≥ 10 μM, although the values found after this concentration were basically at the limit of the detection of the assay. A catecholate, earlier described as 2,3-DHBA, was the only iron-binding ligand in iron-sufficient media (Page & Huyer, 1984). For this reason, the supernatants of growth at all iron concentrations (determined up to 750 μM), were extracted with ethyl acetate, and samples were run on TLC plates. A catecholate compound became visible when the samples were run on the TLC plates. Indeed, this compound migrated at the same pattern as authentic 2,3-DHBA on the TLC plate. However, analyses of the reactions of 2,3-DHBA with ferric citrate and CAS-shuttle solution revealed that 2,3-DHBA (1 mM) formed a purple colour with ferric citrate (10 μg/ml) and did not cause deferration in the CAS-shuttle assay when the concentration of 2,3-DHBA was up to 1 mM. At 1 mM 2,3-DHBA concentration, the blue colour of the CAS-shuttle solution became slightly denser indicating binding of 2,3-DHBA to the CAS-ferric complex without deferrating it. However, increasing concentration of 2,3-DHBA to 10 mM caused an immediate

deferration of the CAS-ferric complex by the formation of a purple colour. On the other hand, decreasing the concentration of 2,3-DHBA to 0.5 mM, produced a greenish-yellowish colour with ferric citrate. Apparently, at less than 0.5 mM concentrations, the colour of the solution was masked notably by the yellowish colour of ferric citrate. When the supernatants of UW from Table-6 that had grown at iron concentrations ≥10 μM were mixed with ferric citrate, no colour change to purple was found with ferric citrate, and the colour of the solutions remained yellowish. This indicated that what was seen in the ethyl acetate fraction may be a slight contribution from 2,3-DHBA. Probably, the concentration of 2,3-DHBA and its derivative siderophores in the culture supernatant fluids markedly decreased, and therefore they no longer formed a visible purple colour with ferric citrate.

When examined spectrophotometrically, the concentrated samples of strain UW grown with increasing iron concentrations gave a broad absorption maximum around 300 nm, although the computerized program of the spectrophotometer did not recognize it as a peak. Furthermore, the resolution of TLC was also tested by using pure samples of 2,3-DHBA (MW= 154) and authentic catechol (1,2-dihydroxy benzene, MW= 110) as a control under the same buffer conditions. It was observed that both compounds migrated together at the front and they were not separated from each other. However, this may have been a problem with the solvent system although it was not well studied. Furthermore, strain P100, defective in known catecholate and hydroxamate type siderophores, had the same pattern in its EA extracts prepared from 10-fold concentrated supernatants.

It was concluded that the ethyl acetate extracted catecholate was not 2,3-DHBA alone, but that some other products must have been in the solution. If 2,3-DHBA was produced alone in iron-sufficient media, how would it support the growth of the organism? In order to answer this question the role of 2,3-DHBA was further investigated in an attempt to see how it may contribute to the growth of the organism.

3.12. Does 2,3-DHBA aid growth of *A. vinelandii* ?

Strains UW, D27, F196, UA1, and P100 were incubated with 500 μM 2,3-DHBA (approximately this amount of total catecholate was normally formed after 1

day growth in iron-deficient Burk's (0 μM Fe) medium) with 1 μM iron (iron-deficient) and 25 μM iron (iron-sufficient) containing Burk's media. The results showed that almost all strains grown in iron-sufficient (25 μM) media did not take up 2,3-DHBA since practically all 2,3-DHBA was found in the medium. This can be shown in Table-7 since the peak A_{310} for the cultures, other than strain UA1 (- 0.1) grown without 2,3-DHBA is exactly equal to the peak A_{310} for the blank. In fact 0.1 unit difference obtained with this strain falls within the range of the experimental error. In the case of the iron-deficient grown cultures the results were essentially the same other than strain D27. In this case, one could add the peak A_{310} for the cultures grown without 2,3-DHBA to that of the blank in order to find the peak A_{310} for the cultures grown with 2,3-DHBA. The unit of A_{310} values obtained with strains UW (- 0.1), F196 (+ 0.2), UA1 (- 0.3), P100 (0.0) were equal to that of the blank within the range of the experimental error. However, in the case of strain D27 catecholate peak at A_{310} was equal to that of the blank medium. These values are also within the error range of the spectrophotometer since some peaks have a wider valley while others have a narrower one. Furthermore, the production of azotobactin by strains UW and F196 appeared to be unaffected in iron-deficient media whether 2,3-DHBA was present or not (Table-7). The protein yield of the strains varied in the presence of exogenously added 2,3-DHBA. In iron-deficient Burk's media strains UW, D27, F196 and P100 increased their total cell protein, 11 %, 49 %, 16 % and 27 %, respectively. Whereas, there was about 13 % decrease in the case of strain UA1. The increase in the protein observed in the case of strain D27 was notable. If somehow 2,3-DHBA was degraded and metabolized by this strain, it would probably account for this increase in the protein content as well as the notable decrease in the A_{310} peak for catecholates. Moreover, the data obtained with strain D27 was repeatable. The reason for this is unclear. On the other hand, in the iron-sufficient medium strains UW, D27, UA1 and P100 decreased their total cell protein, 11 %, 12 %, 22 % and 13 %, respectively. While strain F196 did not change it. Overall, this results may suggest that 2,3-DHBA may cause a slight increase in the total cell protein content when the medium is iron-deficient and a slight decrease when the medium is iron-sufficient. The latter result may seem to be contradictory for its role assigned for

Table-7. Effect of 2,3-DHBA on siderophore production and total cell protein content of the strains.*

	A_{310} (catecholate)							
	1 μM Fe				25 μM Fe			
Strains	with DHBA		without DHBA		with DHBA		without DHBA	
UW	2.3	(255)	0.9	(230)	1.5	(426)	0	(453)
D27	1.5	(324)	1.0	(218)	1.5	(399)	0	(447)
F196	1.7	(199)	0	(172)	1.5	(445)	0	(445)
UA1	2.6	(221)	1.4	(251)	1.4	(374)	0	(456)
P100	1.5	(210)	0	(165)	1.5	(401)	0	(452)
Blank	1.5	(N/A)	0	(N/A)	1.5	(N/A)	0	(N/A)

	A_{380} (azotobactin)			
	1 μM Fe		25 μM Fe	
Strains	with DHBA	without DHBA	with DHBA	without DHBA
UW	0.7	0.9	0	0
D27	0	0	0	0
F196	1.4	1.4	0	0
UA1	0	0	0	0
P100	0	0	0	0
Blank	0	0	0	0

* All strains were grown 24 h at 30 °C at 225 rpm. A_{310} (catecholate) and A_{380} (azotobactin) were determined from the strains grown with and without added 2,3-DHBA (500 μM) under iron-deficient (1 μM Fe) and iron-sufficient (25 μM Fe) Burk's medium. Protein values (μg/ml) are given in brackets next to catecholate values, and the same protein values are also valid for azotobactin values in the corresponding order. N/A: not applicable because no cells were present.

low-affinity iron uptake (Page & Huyer, 1984). However, the data about the variations in the protein content are not considerably great. In conclusion, the production of siderophores appear to continue in the presence of 2,3-DHBA in iron-deficient media. If 2,3-DHBA somehow aided the growth of the mutants, say, by being recycled without being internalized, with a "taxi-cab mechanism" as shown in *Rhodotorula pilimanae* (Carrano & Raymond, 1978) (see Introduction, p. 11), one might still expect cooperative production of siderophores from the siderophore defective mutants as well as the parental strain UW in order to meet the iron demand of the cells. These results suggested that 2,3-DHBA may not be the only iron-chelator found in iron-sufficient cultures of the organism. Furthermore, the ethyl acetate extracted fraction must include a compound at high iron concentrations which should have a phenol-like or reducing structure in order to elicit a positive reaction to the 2,2'-bipyridyl : ferric citrate spray (Krebs *et al.*, 1969).

The effect of 2,3-DHBA on the growth of strain P100 in iron-sufficient (25 μM Fe) Burk's medium containing EDDHA was also investigated. Strain P100 was pregrown in iron-deficient (0 μM Fe) Burk's medium overnight to minimize the iron content. Growth of strain P100 in iron-sufficient (25 μM Fe) Burk's medium plus 50 μg/ml EDDHA, and plus 500 μM 2,3-DHBA pre-ferrated with 25 μM ferric citrate or not pre-ferrated in the media showed that the growth of strain P100 had ceased completely (Fig. 15). This growth inhibition of strain P100 was not seen when the filter sterilized supernatant of the parent strain from iron-deficient growth was included in the medium (5 ml supernatant of strain UW from iron-deficient culture in 100 ml iron-sufficient (25 μM Fe) Burk's medium (Fig. 15). In a way, this experiment also confirmed the result obtained in the plate assay (Fig. 9).

3.13. Analysis of the concentrated supernatant of strain P100

Earlier, it was found that a siderophore activity was present in the supernatants of strain P100 (Fig. 10 and Tables 2 and 3). Moreover, some small amount of catecholate was detected in the supernatant of the cells grown in iron-deficient (1 μM Fe) medium (Table-2). Furthermore, when the supernatant of strain P100 was concentrated 10-fold by freeze-drying in an attempt to increase its

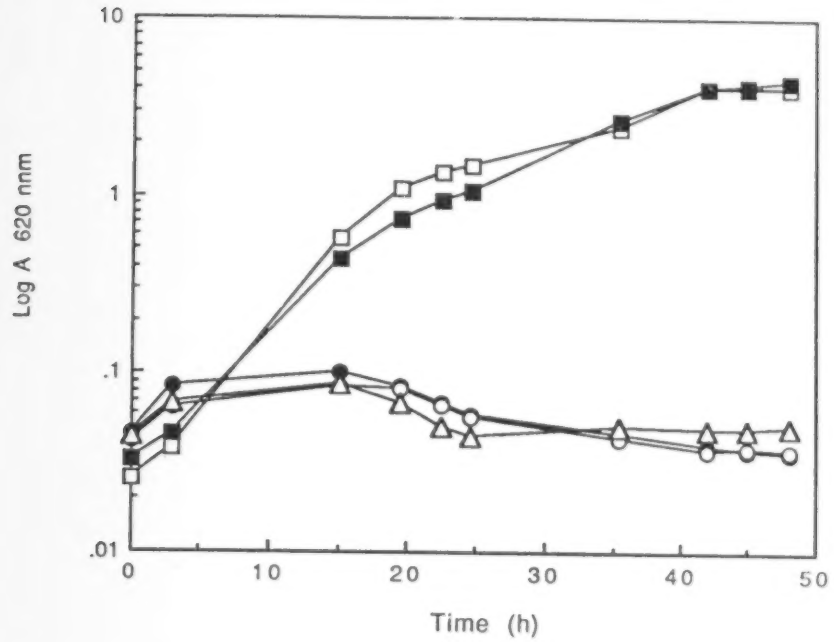


Figure-15. Effect of EDDHA, 2,3-DHBA and iron-deficient (0 μM Fe) supernatant of strain UW on growth of strain P100 (50 μg/ml EDDHA was included in iron-sufficient (25 μM Fe) Burk's medium. (□) No EDDHA; (●) with EDDHA; (○) with EDDHA plus 500 μM 2,3-DHBA; (△) with EDDHA plus 500 μM 2,3-DHBA ferrated with 25 μM ferric citrate; (■) with EDDHA plus 5 ml supernatant of strain UW from iron-deficient (0 μM Fe) grown culture).

siderophore activity and eliminate the possibility of any interference occurred in the assay. Indeed, this process increased the concentration of the catecholate, and when extracted with ethyl acetate, the ethyl acetate extracted fraction contained the catecholate at a higher level. The siderophore activity was present in both the ethyl acetate and the aqueous phases as determined by the CAS-shuttle assay.

A number of general biochemical tests was performed. Ninhydrin reactions of the ethyl acetate and the aqueous fractions were negative indicating that they did not possess a free amine group. There was also no detectable citrate. Moreover, a reducing activity (from Fe^{3+} to Fe^{2+}) accompanied with the formation of a reddish colour was found when ascorbate was replaced with the supernatant of strain P100 in the iron determination assay. This reducing activity was higher when the concentrated supernatants were used. The original supernatant from strain P100 had a transparent colour, but over the time of repeated concentration and other processes, it changed from yellowish to brownish, darkish colours, probably due to oxidation. Moreover, normally when acidified supernatants are scanned spectrophotometrically from 500 nm to 200 nm, usually a peak is obtained around 265 nm, which is called the "end peak" (W.J. Page, personal communication). A similar peak also was obtained when ascorbate was scanned. Furthermore, ascorbate also gave a positive CAS reaction. However, analysis of supernatants from all concentrates with an assay specific for ascorbate did not detect any ascorbate as a product of strain P100.

Furthermore, a pure siderophore, ferroxamine (Desferal®, Ciba-Geigy) was analyzed for the presence of reducing activity and/or in an attempt to see whether this reducing activity is common to other siderophores. Surprisingly, ferroxamine also showed a reducing activity, primarily at acidic pH (pH 2 ≤) (Table-8). This reducing activity in the bipyridyl assay was also observed to occur with prolonged incubation spontaneously under acidic conditions without the aid of any reducing agent. However, no reducing activity was found to occur spontaneously when the pH of the solution in the assay was above pH 3 within 5.5 h (Table-8). It appeared that the concentrated supernatant of strain P100 and the siderophore ferroxamine accelerated this reducing activity in the assay. However, with prolonged incubation of the reduction assay mixtures, the red colour formed intensified at 20.5 h (Table-

Table-8. Comparison of spontaneous and siderophore-mediated reducing activities at various pH†.

Incubation Time:	A_{530} nm 10 min			5.5 h			20.5 h		
pH	Control*	+ P100**	+Desferal‡	Control*	+ P100**	+Desferal‡	Control*	+ P100**	+ Desferal‡
1	0.036	0.254	0.253	0.152	0.880	0.408	0.253	1.170	0.658
2	0.098	0.207	0.269	0.423	0.612	0.274	0.956	0.871	0.288
3	0.038	0.071	0.056	0.181	0.085	0.070	0.418	0.129	0.076
4	0.021	0.050	0.030	0.036	0.060	0.026	0.056	0.089	0.027
5	0.022	0.048	0.027	0.028	0.056	0.024	0.061	0.080	0.025
6	0.021	0.056	0.025	0.011	0.050	0.014	0.036	0.088	0.035
7	0.023	0.062	N/D	0.008	0.047	N/D	0.031	0.093	N/D
8	0.028	0.055	0.027	0.011	0.051	0.015	0.030	0.084	0.032
9	0.020	0.052	0.026	0.011	0.055	0.023	0.013	0.077	0.033
10	0.019	0.057	0.031	0.021	0.061	0.033	0.26	0.085	0.049

† Reducing activities were determined by replacing ascorbate with the indicated compound in the iron determination assay. Absorbances were measured at 530 nm against distilled water with the time intervals shown above. The assay was carried out in the presence of 10 µg/ml $FeCl_3$. The red color formed was slightly or not visible by the naked eye at absorbances ≤ 0.100

* Spontaneous reduction with no reducing agent.

** With highly concentrated supernatant (about 50-fold) of strain P100 from iron-deficient (0 µM Fe) Burk's medium.

‡ Desferal (Ciba-Geigy), pure siderophore Deferrioxamine B from *Streptomyces pilosus* was added to 1 mg/ml.

8) and longer (data not shown) incubation time. Since the bonding between a siderophore and Fe^{3+} is almost always achieved through an oxygen atom, one could conceive that during the formation of the siderophore-ferric complex, one electron from oxygen is transferred to Fe^{3+}, resulting the reduction of Fe^{3+} to Fe^{2+}. This reducing activity was previously shown to occur under acidic conditions in the presence of excess catechol (Mentasti *et al.*, 1976; Rioux *et al.*, 1988). However, 2,3-DHBA, when tested, immediately bound to ferric ion with the formation of a purple colour in the reduction assay.

3.14. Gel exclusion chromatography of the concentrated supernatant of strain P100

The 10-fold concentrated supernatant was run through a gel exclusion chromatography column and the eluted sample was concentrated by freeze-drying. The final volume was adjusted to about a 50-fold concentration of the original sample. A 1 ml sample from this solution was loaded onto a second column. This resulted in the formation of two major peaks as obtained by the CAS-shuttle assay: the first early peak (Peak 2.1) had a higher CAS activity than the second peak (Peak 2.2) (Fig. 16). Later, both peaks were concentrated as before and loaded separately onto the column. All fractions were concentrated in a small volume, and they were assayed qualitatively for the presence of catecholate, glucose, reducing activity as well as CAS-shuttle activity (Table-9). The first peak came out as one single peak called Peak 3.1 (Fig. 17). It included CAS activity and reducing activity. Peak 3.1 was practically free from glucose and catecholate (Table-9). On the other hand, the second major peak resulted into two smaller peaks: namely Peak 3.2.1 and Peak 3.2.2 (Fig. 18). Peak 3.2.1 had relatively less reducing activity and CAS activity while it was also free from glucose and catecholate. However, Peak 3.2.2 had all the four at highest level: namely reducing activity, CAS-shuttle activity, glucose and catecholate (Table-9).

The results apparently showed that there were at least two different siderophore activities in the supernatant of iron-deficient grown strain P100. Peak 3.1 and Peak 3.2.2 were quite distinct from each other. Peak 3.2.1 may have been related to peak 3.1 or it may have also included another siderophore. Because, the

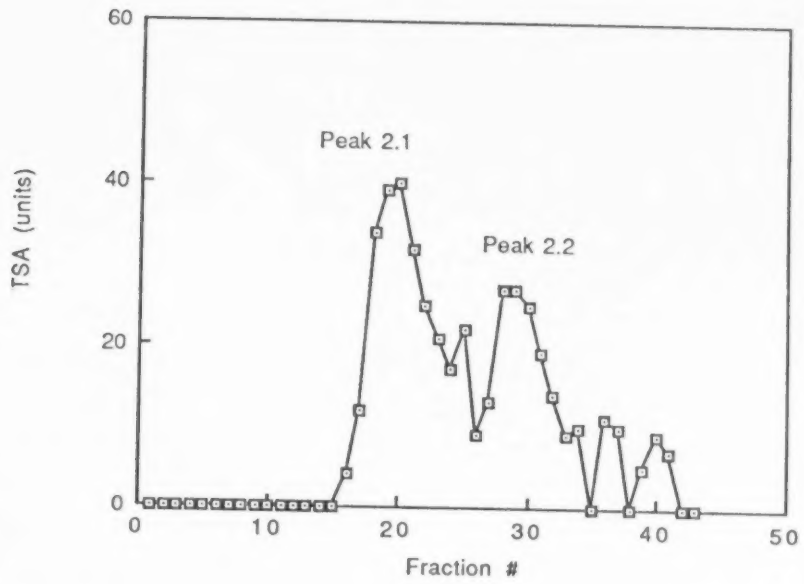


Figure-16. Elution profile of Peak 2.1 and Peak 2.2 (from highly concentrated supernatant of strain P100). The sample was eluted through a gel exclusion chromatography column containing Bio-Gel P-2. The size of the column was 1 cm diameter by 32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column about 0.2 ml/min. Each fraction had about 1 ml volume. Fractions were analyzed by CAS-shuttle assay.

Table-9. Characterization of the siderophore peaks of strain P100 obtained from a gel-exclusion chromatography column[#].

Sample	Reducing activity[†]	CAS shuttle[¶]	Glucose[x]	Catecholate[xx]
Peak 3.1[*].	++	++++	-	±
Peak 3.2.1[*].	+	++	-	-
Peak 3.2.2[*].	++++	++++	++++	++++
Aqueous phase (alone)[‡]	+	+++	++++	±
Peak AQ				
#11-#16	±	+++	-	N/D
#17-#28	±	+++	+++	N/D
Ethyl acetate phase (alone)[‡]	+++	+++	±	+++
Peak EA-1 (#18-#23)	++	+++	N/D	±
Peak EA-2 (#24-#29)	++	++++	N/D	±

[#] Iron-deficient (0 μM Fe) grown culture supernatant of strain P100 was concentrated before gel-exclusion chromatography.

[¶] All evaluations of the assays were relative to each other in terms of intensity of colour change as well as rapidity of the reaction as determined qualitatively by the naked eye. Each (+) sign refers to a degree of positiveness while (-) sign refers to a negative reaction, and (±) sign refers to a slightly positive or unclear reaction.

[†] Reducing activity was detected by replacing ascorbate with the concentrated fraction sample in the iron determination assay.

[*] Fractions from the 2nd loading of the highly concentrated (about 50-fold) supernatant of strain P100 onto the column gave two major peaks: the first one was re-loaded and gave one peak, called peak 3.1, and the second peak was re-loaded and gave two smaller peaks, called peak 3.2.1 and peak 3.2.2 respectively.

[‡] Highly concentrated supernatant of strain P100 from 2nd elution was extracted 7-times with 2-3 volumes of ethyl acetate. Ethyl acetate was evaporated thoroughly from both the ethyl acetate and aqueous phases and the ethyl acetate phase was dissolved in original volume of distilled water. The pH of both phases were adjusted to about 6.0 before assaying. They were later loaded into the column.

[x] Glucose determined by using an enzyme kit from Sigma. Colour formation was determined qualitatively by the naked eye.

[xx] Barnum assay (1977) was performed. Formation of colour was determined qualitatively by the naked eye.

N/D: Not determined.

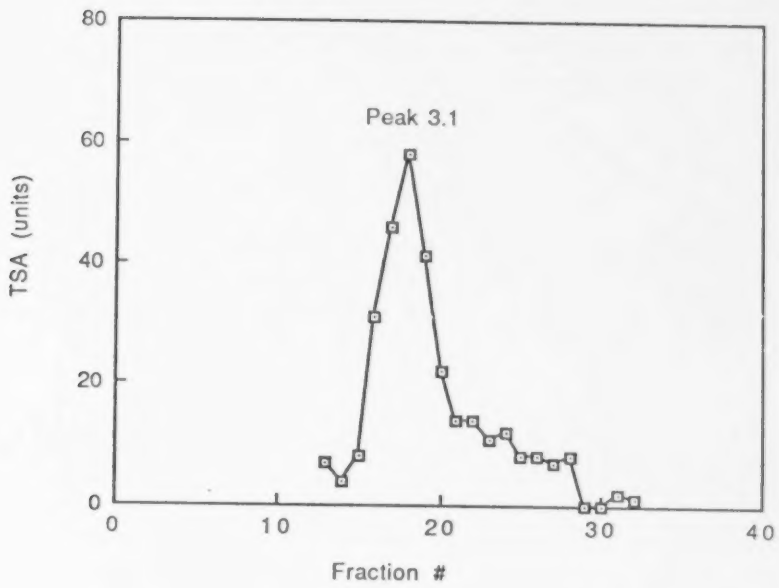


Figure-17. Elution profile of Peak 3.1 (from highly concentrated supernatant of strain P100). The sample was eluted through a gel exclusion chromatography column containing Bio-Gel P-2. The size of the column was 1 cm diameter by 32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column about 0.2 ml/min. Each fraction had about 1 ml volume. Fractions were analyzed by CAS-shuttle assay.

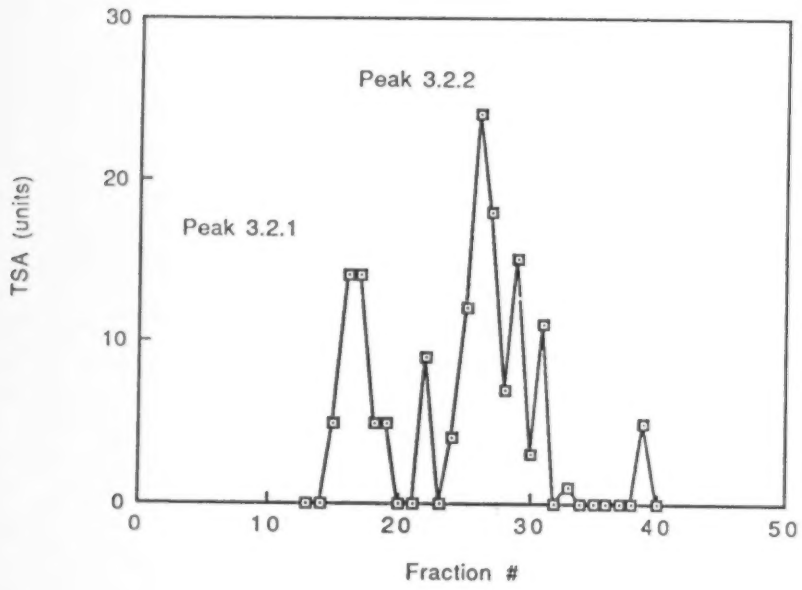


Figure-18. Elution profiles of Peak 3.2.1 and Peak 3.2.2 (from highly concentrated supernatant of strain P100). The sample was eluted through a gel exclusion chromatography column containing Bio-Gel P-2. The size of the column was 1 cm diameter by 32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column about 0.2 ml/min. Each fraction had about 1 ml volume. Fractions were analyzed by CAS-shuttle assay.

gel exclusion chromatography separated them on the base of molecular size, it was possible that Peak 3.2.2 had a MW that was close to that of glucose (180) whereas Peak 3.1 had the biggest MW among them, and Peak 3.2.1, if it was another siderophore, its molecular size would fall in between these two MWs.

3.15. Gel exclusion chromatography of the ethyl acetate and aqueous phases of the concentrated supernatant of strain P100

This highly concentrated supernatant of strain P100 (about 50-fold) was extracted thoroughly with ethyl acetate, and the ethyl acetate extracted phase and the aqueous phase were analyzed separately. Both phases were also loaded onto another gel exclusion column. The results in Table-9 showed that practically all contaminating glucose remained in the aqueous phase while all the catecholate passed into the ethyl acetate phase. Elution of the aqueous phase through the column showed that the aqueous phase came out as a single peak, called Peak AQ (Fig. 19), and the early fractions of this peak came out free of glucose, while in the later fractions glucose was associated with the siderophore activity (Table-9). This indicated that the presumed siderophore in the aqueous phase had a MW somewhat larger than that of glucose. On the other hand, gel exclusion chromatography of the ethyl acetate phase resulted in two peaks (Fig. 20). The first peak (Peak EA-1) came out as a flat plateau, followed by a sudden sharp rise of the second peak (Peak EA-2). Essentially, both peaks were very similar in many ways other than the size. From Table-9 it appeared that the presumed siderophores in the ethyl acetate phase had MWs smaller than that of Peak AQ, perhaps near that of glucose. However, Peak EA-2 was apparently of the smallest MW. In conclusion, the gel exclusion chromatography data obtained without ethyl acetate extraction in section **3.14** was confirmed after ethyl acetate extraction of the sample in this section. Essentially observation of three different peaks corresponding to three different siderophore activities were substantiated with two different experimental approaches.

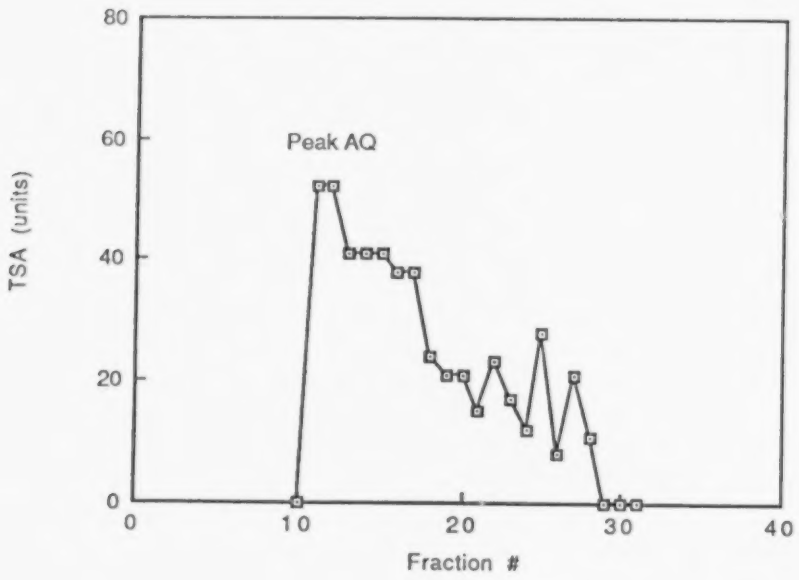


Figure-19. Elution profile of Peak AQ (from aqueous phase of ethyl acetate extracted, highly concentrated supernatant of strain P100). The sample was eluted through a gel exclusion chromatography column containing Bio-Gel P-2. The size of the column was 1 cm diameter by 32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column about 0.2 ml/min. Each fraction had about 1 ml volume. Fractions were analyzed by CAS-shuttle assay.

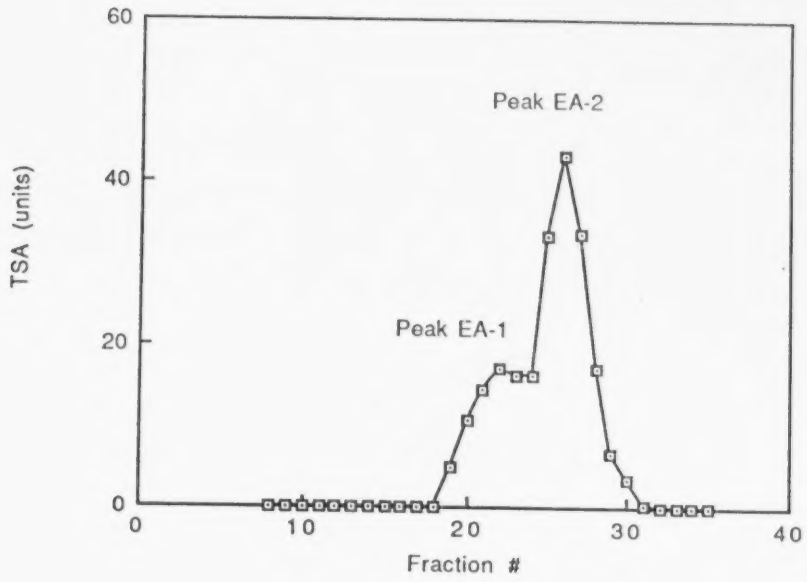


Figure-20. Elution profiles of Peak EA-1 and Peak EA-2 (from ethyl acetate phase of ethyl acetate extracted highly concentrated supernatant of strain P100). The sample was eluted through a gel exclusion chromatography column containing Bio-Gel P-2. The size of the column was 1 cm diameter by 32 cm height. The column was operated under about 70 cm H_2O pressure. Flow rate of the column about 0.2 ml/min. Each fraction had about 1 ml volume. Fractions were analyzed by CAS-shuttle assay.

4. DISCUSSION

The genome of *Azotobacter vinelandii* was mutagenized with a Tn*5*-construct in an attempt to obtain mutants defective in iron uptake. The mutagenesis resulted in a number of mutants. Although the process is called "conjugation", it does not appear to be a normal conjugation because of the absence of *tra* genes, the length of time it takes to occur and its inefficiency. It is likely that this system of conjugation may be missing some components. However, at the moment not much is known about it other than that it is not a transformation mechanism since the process is DNAse resistant and the competent as well as noncompetent cells of *Azotobacter vinelandii* gave about the same frequencies of transconjugants when mated with the donor *E. coli* strain.

The siderophore-defective mutants developed in this study appear to bring in new insights into understanding the operation of iron metabolism in *Azotobacter vinelandii*. Under iron-deficient conditions it is obvious that siderophores support the growth of the organism. Loss of the pyoverdin-type siderophore azotobactin seems to have little effect on the growth of *A. vinelandii* under iron-deficient conditions as observed from the turbidity and protein measurements of the mutant strains D27 and UA1 (Fig. 6 and Table-1). However, loss of catecholate siderophores seems to have a more serious effect on the growth of *A. vinelandii* under iron-deficient conditions, as observed with strain F196. This effect is less pronounced in turbidity measurements under iron-deficient conditions, due to interference stemming from the yellow-green colour of azotobactin. Therefore, measurements of the protein content provide a clearer picture of the effect of iron-deficiency. Catecholate siderophores are known to be low-affinity chelators of iron, providing bidentate or tetradentate coordination (Page & von Tigerstrom, 1988). On the other hand, loss of both of these types of siderophores affected the growth of the organism noticeably under iron-deficient conditions as can be seen from strain P100.

Generation of strain P100 is of importance since it is deficient in the known catecholate and the pyoverdin siderophores. However, it produced orange halos when grown on CAS plates and gave a positive CAS-shuttle reaction indicating that

there was still siderophore activity present in the iron-deficient supernatants of the organism. However, the earlier results suggested that there was a non-conventional siderophore in the supernatants of strain P100, i.e. neither hydroxamate nor catecholate, since it could not be detected with conventional techniques. Presently, the only detection technique appears to be the CAS assay of Schwyn & Neilands (1987).

The siderophore activity of strain P100 appears to have a relatively low iron-chelation activity since EDDHA at 50 μg/ml had a higher affinity for iron than the siderophore of strain P100. Thus, EDDHA was an effective inhibitor of the growth of strain P100 in both iron-deficient conditions on solid media (Fig.9) and iron-sufficient conditions in liquid media (Figs. 7,8). The inhibitory effect of EDDHA can be overcome if the total siderophore activity in solution is increased, either by concentrating the supernatant of strain P100 or feeding the organism with the siderophores from other siderophore producing strains or by increasing the stored iron content in bacterioferritin. The iron-storage protein, bacterioferritin of *Azotobacter vinelandii* (Jiudi *et al.*, 1990; Steifel & Watt, 1979) apparently can store a large amount of iron (Fig. 13) and this reservoir can help strain P100 survive efficiently when the iron is scarce in the environment.

The siderophore activity of strain P100 can also internalize iron from iron-containing minerals, as described for the parental strain UW and azotobactin defective strain UA1 (Page & Huyer, 1984). However, the efficiency of the siderophore activity of strain P100 seems to be relatively low (Table-4). Furthermore, growth of strain P100 under nitrogen-fixing conditions at high aeration was impaired. Being devoid of its major siderophores, strain P100 seemed to grow poorly in nitrogen-free Burk's medium (Table-5). Increasing the iron concentration from zero to 1 μM decreased the doubling time of strain P100 but negligible increase was observed when the iron concentration reached 25 μM. Under iron-sufficient conditions, growth rates of the strains were very similar to each other. However, under these conditions, a low level of siderophore activity was apparent in the supernatants of all mutants, indicating that the iron from the medium was not available freely, probably because of its oxidation to Fe^{3+} and low solubility.

Observation of strongly iron-repressible bioluminescence in F196 indicated that the *luxAB* genes of the luciferase enzyme carried by the transposon had found a highly iron-sensitive promoter for its expression. Accompanying this feature was the disappearance of catecholate siderophores, suggesting that the *luxAB* genes from the transposon may have been using the promoter and/or operator sequence in the upstream sequence of the biosynthetic genes essential for the synthesis of these siderophores. Indeed it has recently been shown that another Gram-negative organism *Escherichia coli*, the original donor host of Tn*5luxAB*, has a highly sensitive iron box acting as an operator for the *Fur* -repressor protein (De Lorenzo *et al.*, 1987; Calderwood & Mekalanos,1988; Wee *et al.*, 1988). Therefore, this assumption for the location of the insert should be reasonable. On the other hand, no bioluminescence was detected with the strain D27 under iron-deficient and iron-sufficient conditions (Table-2). In this strain possibly the insertion of the transposon was in the reverse orientation, the upstream region of the luciferase casette did not have an appropriate promoter for its expression, or the repressor bound to the operator sequence was not responsive to iron depletion.

Currently, very little is known about the organization of the genes involving in the biosynthesis and regulation of the gene products for siderophore production in *Azotobacter vinelandii*. However, the data obtained from this study suggests that the genes required for biosynthesis of the 2,3-DHBA derivative siderophores, azotochelin and aminochelin, are organized in an operon since in strain F196, and its siblings, the Tn*5luxAB* insertion has been shown to omit the production of all the siderophores derived from 2,3-DHBA. This was not surprising since the operator and/or promoter site may now be regulating the expressing of the *luxAB* genes as mentioned above. Furthermore, this is not the only operon organized siderophore system since the biosynthetic genes for the aerobactin siderophore of *E. coli* has also been reported to form an operon (De Lorenzo & Neilands, 1986; Neilands, 1989). Moreover, the genes involved in the biosynthesis of pyoverdin-type siderophores of various *Pseudomonas* strains have been reported to cluster in operons (Loper *et al.*, 1984; Ankenbauer *et al.*, 1986; Hohnadel *et al.*, 1986; Magazine *et al.*, 1986). In addition, the IROMP for transport of 2,3-DHBA-derivative siderophores in these mutants may or may not be organized within this

operon since their IROMPs were still functional. Siderophores from other azotobactin-defective strains, D27 and UA1, appeared to be transported efficiently into strain P100 in order to promote its growth (Fig. 9).

Earlier Glick *et al.* (1988) obtained siderophore-defective mutants of *A. vinelandii* by means of chemical mutagenesis. They obtained azotobactin-minus mutants (Flu^-), most of these were also missing catecholate siderophores and some were even missing the IROMPs. They also obtained negative results in the CAS assay with the double siderophore-defective mutants. They concluded that the syntheses of azotobactin and azotochelin siderophores were coupled. However, the data presented here contradict their conclusion since azotobactin-defective and catecholate-defective mutants were obtained independently and catecholate production continues in the absence of azotobactin. It is probable that their data resulted from mutations at more than one site on the chromosome, and their conclusions, therefore, are not valid. On the other hand, azotobactin was overproduced in the absence of catecholate siderophores in strain F196. This may be due to the function of this siderophore rather than a result of the mutation. Usually catecholates solubilize iron from mineral sources while azotobactin prefers to chelate soluble iron (Page & Huyer, 1984; Page & von Tigerstrom, 1988). Thus, azotobactin is overproduced once the production of catecholates falls short in order to capture iron from insoluble minerals in an attempt to promote the growth. Overall, transposon mutagenesis appears to be a much more effective and controlled way of mutagenesis than chemical mutagenesis. Furthermore, transposon mutagenesis overcomes the problems of obtaining mutants in *A. vinelandii* stemming from the multicopy (≥40) chromosome of the organism (Punita *et al.*, 1989; Sadoff *et al.*, 1979).

From the gel exclusion chromatography data it appears that there are three new siderophores are present in the iron-deficient culture fluid of *Azotobacter vinelandii*. These results obtained in the gel exclusion chromatography are confirmed with two different approaches, namely elution of samples before and after ethyl acetate extraction. The presence of these new siderophores in the wild type strain normally remains undetected. With the removal of the major siderophores, it is likely that these new siderophores are overproduced. Analysis

of the ethyl acetate extracted, highly concentrated supernatants of strain P100 from iron-deficient growth showed that siderophore activity was present both in the ethyl acetate phase as well as in the aqueous phase. Thus, use of this strain in this study made it easier to detect their presence. By using gel exclusion chromatography in combination with the general siderophore assay, CAS-shuttle and ethyl acetate extraction made it easier to detect more siderophores from the organism that has already three known major siderophores. Gel chromatography of the highly concentrated supernatant of strain P100 showed that there were three peaks as determined by their CAS activity. Because each peak corresponded to a different MW, one would expect three more different siderophores in the iron-deficient supernatant of strain P100. The aqueous phase appears to include a structurally nonconventional siderophore while two catecholate positive substances, not likely to be 2,3-DHBA, were present in the ethyl acetate phase. The argument whether these two catecholate peaks observed in the ethyl acetate phase (EA-1 and EA-2) are related to 2,3-DHBA derived siderophores can also be ruled out since these two peaks as well as Peak-AQ have negative ninhydrin reactions. Among the two 2,3-DHBA derived siderophores, aminochelin has a free amine group as a part of its putrescine moiety, and if aminochelin were produced together with azotochelin or alone, its presence would give a positive ninhydrin result.

The concentrations of these siderophores are apparently so low that normal measurement of their concentration remains below or at the detection limit of the Barnum assay (1977). At the moment their concentrations in unconcentrated solutions are too little to quantify even with the CAS-shuttle assay. However, they can be purified essentially free of the impurities although glucose may be found in small quantities in the latter fractions of the aqueous phase but not in the early fractions obtained from the elution of this phase. The three new siderophore activities shown here are probably minor iron-chelating compounds of the organism. They may be acting as emergency siderophores in the absence of the major iron-chelators. Determination of their structure might facilitate their quantification.

From Table-9, it can be seen that after ethyl acetate extraction of the highly concentrated supernatant of strain P100 gives a catecholate positive reaction in a

small volume. However, after elution thorough the column an unclear or negative catecholate reaction is obtained due to dilution of the sample. However, the CAS activity is affected either little or not at all. Furthermore, TLC analysis of all culture fluids shows the presence of a catecholate-type compound leading in the front line. This compound is visible even when the medium iron concentration is extremely high, at least 750 μM, and no CAS activity is detectable. However, this compound may be or may not be a siderophore or it may also be a degradation product of the non-2,3-DHBA-derivative catecholate compounds.

Furthermore, association of a reducing activity with the presence of the siderophore activity is of significance. Earlier, it was shown that in acidic conditions, Fe^{3+} is reduced to Fe^{2+} in the presence of excess catechol compounds (Rioux *et al.*, 1983). In this reaction two Fe^{3+} is reduced to two Fe^{2+} by the vicinal hydroxyl groups on the catechol molecule with the oxidation of catechol to *o* -benzoquinone (Mentasti *et al.*, 1976). This Fe^{2+} is known to bind to 2,2'-bipyridyl with the formation of a red colour (Barton, 1965). The observed reduction of Fe^{3+} to Fe^{2+} in the presence of siderophores might indicate that siderophores may also be involved in the reduction of ferric to ferrous iron. However, it is not known whether this reduction event may take place intracellularly. In the bipyridyl assay, under acidic conditions, ferric ion is gradually reduced to the ferrous form without the aid of siderophores. However, with siderophores, this transition process is accelerated, although not all siderophores may act the same way. This protonated reduction mechanism under acidic conditions has been suggested to occur naturally in the periplasmic space where a lower pH is maintained that favors reduction of iron (Stock *et al.*, 1975). Furthermore, reduction of ferric-siderophore complex with decreasing pH is favored greatly (Lee *et al.*, 1985). This previous data is confirmed in Table-8. However, the actual mechanism of this reduction remains ambiguous.

The discovery of more siderophores in the iron-deficient supernatants of strain P100 shows that iron uptake in *Azotobacter vinelandii* is complicated, with many iron-chelators produced simultaneously but at different proportions. Several different non-conventional siderophores have been shown in a variety of bacterial species recently. Examples include, citrate from *Bradyrhizobium japonicum*

(Guerinot *et al.*, 1990), rhizobactin from *Rhizobium meliloti* (Smith & Neilands, 1984, 1987; Smith *et al.*, 1985), staphyloferrin from *Staphylococcus hyicus* (Meiwes *et al.*, 1990), alpha-hydroxyisovaleric acid from *Proteus mirabilis* (Evanylo *et al.*, 1984). Demonstration of more siderophores in iron-deficient supernatants of *Azotobacter vinelandii* should not be surprising since mixing the right amounts of catecholate and azotobactin siderophores has never resulted in recovery of the full siderophore activity (Knosp *et al.*, 1984; Page & von Tigerstrom, 1988; Page & von Tigerstrom, unpublished data). This was because not all the siderophores from the organism had been identified at that time.

In the past the role of siderophores in high-affinity iron uptake has been well studied. However, their possible roles in low-affinity iron uptake remained unclear. How iron is taken up under iron-rich conditions has remained almost a mystery. Iron as a charged molecule that will not normally pass through the cell membrane. In the case of *Bacillus megaterium* it was shown that the siderophore-ferric complex requires an active transport mechanism and this uptake is blocked when the energy metabolism is blocked (Davis & Byers, 1971; LeBlanc & Lankford, 1970). More recently, it has been shown that ATP is indirectly involved in the uptake of the ferric-siderophore complex into *Neurospora crassa* (Winkelman, 1986). Here, ATP maintains a polarized membrane; with decreased ATP levels the membrane is depolarized, and the transport of the complex is inhibited. Overall the transport of the ferric-siderophore complex across the membrane appears to be a receptor and energy-dependent process (Braun, 1990). The results presented here suggest that siderophores may also be produced when iron concentrations are high and apparently sufficient to suppress their production. Under these conditions their presence can only be demonstrated with sensitive methods. Here, concentration of the samples showed that siderophore activity can be increased. The CAS-shuttle assay used in this study seems to be the most sensitive assay so far since specific chemical assays fail to detect after some reduction in the concentration of siderophores.

The suggestion that siderophores play a role in low-affinity iron uptake does not necessarily reject their inducibility under iron-deficient conditions since even with 750 μM ferric citrate after 24 h growth, their presence was evident in the

supernatant of strain P100 that is deficient in the production of high-affinity iron-chelators. Furthermore, practically no soluble iron was found in the culture fluids after this time even when the initial concentration of iron was 750 μM. This may also compel the cell to continue to produce more siderophores. Moreover, it is possible that the newly discovered siderophores are overproduced by strain P100 since they are the only remaining iron-chelators after successive round of mutageneses during the generation of this strain. It is also possible that all siderophores may exist in relatively low quantities during growth on high-iron media although their relative ratios vary. As a matter of fact, interpretation of low-affinity iron uptake in this manner, may also be seen in the expression of other cellular compounds, such as the ß-galactosidase enzyme of *Escherichia coli* which is present at a level of not more than a few molecules normally and introduction of lactose into the medium as a sole carbon source dramatically increases its copy number (Mathews & van Holde, 1990). However, the argument is almost exactly the reverse of the present, case since iron is an essential element while lactose can be dispensable in the presence of glucose. For this reason, one could expect syntheses of siderophores to be continuous, however, their repression would most likely be relative depending on the available iron. Here, one could also assign a role to siderophores of strain P100 being specific for low-affinity iron uptake since they fail to grow on iron-sufficient as well as iron-deficient media when a strong chelator is present. However, this was totally dependent on the stored iron concentration and once the supernatant from iron-deficient grown strain P100 was concentrated, its CAS activity, i.e. its affinity for Fe^{3+} increased, and it more efficiently deferrated the ferric-CAS complex.

Iron-chelators consisting of 2,3-DHBA linked to a specific amino acid have been shown to be present in several bacterial species in nature. Examples are 2,3-dihyroxybenzoyl (DHB)-glycine in *Bacillus subtilis* (Ito & Neilands, 1958), DHB-serine in *Escherichia coli* (Brot *et al.*, 1966; O'Brien *et al.*, 1969), *Aerobacter aerogenes* (O'Brien *et al.*, 1969) and *Salmonella typhimurium* (Wilkins & Lankford, 1970), di-DHB-lysine (Corbin & Bullen, 1969) and DHB-putrescine in *A. vinelandii* (Page & von Tigerstrom, 1988). Although these compounds have been shown to be involved in iron uptake, the role of 2,3-DHBA alone in iron

uptake remains marginal. Since the binding of Fe^{3+} to CAS is apparently much stronger than that of 2,3-DHBA. Therefore, if 2,3-DHBA was the only iron chelator, it would have to be abundant enough to meet the demand in the medium. At 10 mM 2,3-DHBA can efficiently deferrate the CAS-ferric complex while at 1 mM it remains inefficient. The total amount of catecholate formed after 24 h incubation in iron-deficient grown *A. vinelandii* supernatant is often around 0.5 mM, and that is mainly the siderophores derived from 2,3-DHBA rather than itself. This amount of catecholate can efficiently deferrate the CAS-siderophore complex because of the contribution of the siderophores derived from 2,3-DHBA. If all catecholate was 2,3-DHBA, no doubt the iron uptake would be inefficient. In this sense, the role of 2,3-DHBA in iron uptake may be similar to the role of 2,3-dihydroxy-*N*-benzoyl-L-serine (DBS) that is a degradation product of enterobactin. DBS is released into the medium following the uptake of ferric-enterobactin complex, and it plays a considerably less efficient role in iron uptake (Rosenberg & Young, 1974). On the other hand, the amount of catecholate obtained in supernatant of strain P100 is at the detection limit of the catecholate assay but it can efficiently deferrate the CAS-ferric complex. Therefore, the catecholate found in the supernatant of strain P100 is unlikely to be 2,3-DHBA. Earlier demonstration of this compound in the iron-sufficient supernatant of *A. vinelandii* may have resulted from the non-specificity of the spray reagent since ferric chloride : 2,2'-bipyridyl solution was originally designed to detect phenols, vitamin E and other reducing compounds (Krebs *et al.*, 1969). Therefore, it is possible that what was described as 2,3-DHBA previously (Page & Huyer, 1984) could also include the ethyl acetate extracted peaks shown here. This is also supported by the reduction activity of their presumed phenolate or catecholate structures.

Overall, the data concerning acquisition of iron from iron-rich media present a dynamic interpretation of iron uptake without disregarding the inducibility of siderophores under low-iron conditions. However, their degree of expression appears to be inversely proportional to the iron demand of cells. Although the supporting data derives from an organism that is often chronically hungry for iron, it is possible that more examples from other organisms are also present. For example, an hydroxamate-type siderophore, hadacidin produced by several species

of *Penicillium* (Dulaney & Gray, 1962) is unusual in that it is produced into a medium not iron-deficient (Emery, 1974). A recent example, production of an iron-chelator, 3,4-DHBA by *Azomonas macrocytogenes* and *Azotobacter paspali* (Collinson *et al.*, 1987) although 3,4-DHBA was not considered a true siderophore because of its continued production with increased iron concentrations in the media. However, there was an apparent depression in the production of this compound when the added iron concentration was increased from zero to 1.8 μM; the production of 3,4-DHBA dropped from 0.95 nmol/μg protein to 0.49 nmol/μg protein (about 50 % decrease). Nevertheless, with further increases in iron concentration caused little but continuous depression on the production of this compound.

Under normal conditions the siderophores of strain P100 have much lower affinity for Fe^{3+} than the known hydroxamate and catecholates siderophores since the siderophore activity and/ growth of strain P100 can be blocked with EDDHA under iron-sufficient as well as iron-deficient media. However, these seemingly low-affinity iron-chelators can deferrate the ferric-CAS complex, and the deferration activity can also be increased by concentrating the supernatant of strain P100. Therefore, one could presume that expression of this low-affinity iron-chelating activity under iron-rich conditions should be responsible for acquisition of iron from an iron-rich environment.

It is possible that these new siderophores are overproduced in strain P100 since they are the only iron-chelators remaining after removing the known siderophores of the parental strain UW with succeeding mutageneses. Indeed, because of this overproduction, strain P100 can internalize higher amounts of iron than the parent strain when grown in high iron-containing media. Demonstration of low siderophore production under iron-rich conditions may be attributable to low solubility of iron under normal conditions, i.e. aerobic environment and neutral pH. However, in the continuous production of siderophores under iron-rich conditions even minute amounts must contribute not only to acquisition of iron but also to deposition of mass quantities of this element within the cell. Operation of such mechanism in microbial species should broaden our understanding of low-affinity iron uptake systems.

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